DEVELOPMENT AND VALIDATION OF NEWER ANALYTICAL METHODS FOR THE ESTIMATION OF TAPENTADOL HYDROCHLORIDE IN BULK AND IN TABLET DOSAGE FORM BY UV - VISIBLE SPECTROPHOTOMETRY AND HPTLC

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In partial fulfillment for the award of Degree of

MASTER OF PHARMACY

(Pharmaceutical Analysis)

Submitted by

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(Accredited by "NAAC" with a CGPA OF 2.74 on a four point scale at "B" Grade)

MELMARUVATHUR - 603 319

MAY 2012

CERTIFICATE

This is to certify that the research work entitled "**DEVELOPMENT AND VALIDATION OF NEWER ANALYTICAL METHODS FOR THE ESTIMATION OF TAPENTADOL HYDROCHLORIDE IN BULK AND IN TABLET DOSAGE FORM BY UV - VISIBLE SPECTROPHOTOMETRY AND HPTLC"** is submitted to The Tamil Nadu Dr. M.G.R. Medical University, Chennai in partial fulfillment for the award of the Degree of the **MASTER OF PHARMACY** (Pharmaceutical Analysis) was carried out by **NARENDRA BUDDI (Register No. 26106126)** in the Department of Pharmaceutical Analysis under my direct guidance and supervision during the academic year 2011 –2012.

Place : Melmaruvathur

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NARENDRA BUDDI

Dedicated to

My

Parents and

friends

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LIST OF ABBREVIATIONS USED

%	-	Percentage
% RSD	-	Percentage Relative Standard Deviation
µ	-	Micron
µl	-	Microlitre
ng	-	Nano gram
°C	-	Degree Celsius
TAP	-	Tapentadol Hydrochloride
Gms	-	Grams
ICH	-	International Conference on Harmonisation
IR	-	Infra Red
LOD	-	Limit of Detection
LOQ	-	Limit of Quantitation
mg/ tab	-	Milligram Per tablet
min	-	Minute
ml	-	Millilitre
ml/ min	-	Millilitre/Minute
nm	-	Nanometre
pH	-	Negative Logarithm of Hydrogen ion Concentration
HPTLC	-	High Performance Thin Layer Chromatography
rpm	-	Rotations Per Minute
SD	-	Standard Deviation
SE	-	Standard Error
MBTH	-	3-Methyl-2-Benzothiazolinone Hydrazone Hydrochloride Monohydrate
CI	-	Confidence Interval
RSS	-	Residual Sum of Square
µg/ ml	-	Micro gram/ Millilitre
ng/ µl	-	Nano gram/ Microlitre
USA	-	United States of America
EC	-	European Commission

Introduction

1. INTRODUCTION

1.1 INTRODUCTION TO ANALYTICAL CHEMISTRY (P C Kamboj, 2003. Annees A. Siddiqui, 2006)

The pharmaceutical analysis defined as “the branch of practical chemistry which deals with the resolution, separation, identification, determination and purification of a given sample of a medicine, the detection and estimation of impurities, which may be present in drug substance (or) given sample of medicine”.

The substance may be a single compound or a mixture of compounds and may be in the form a tablet, pill, capsule, ampoule, liquid, mixture or an ointment.

The quality control tests involve methods which embrace chemicals, physio - chemical instrumental, microbiological (or) biological procedures.

The pharmaceutical analysis deals with the subject of determining the composition of material in terms of the elements or compound (drug) present in the system.

Any type of analysis involves two steps

- Identification (qualitative)
- Estimation (quantitative)

In qualitative analysis, a reaction is performed in such a way as to indicate the formation of a precipitate, a change of a colour, the dissolution of a precipitate/ complex formation and the evaluation of a gas.

Quantitative analysis is performed ordinarily through five steps. They are sampling, dissolution, precipitation, measurement and calculation.

Method of assay

It indicates the quantitative determination of principal ingredients of the official substances and in preparations.

Qualitative analysis

This is practiced in order to establish the composition of a naturally occurring or artificially synthesized/ manufactured substance.

Qualitative analysis

I. Chemical Methods

a) Titrimetric analysis

b) Gravimetric analysis

c) Gasometric analysis

II. Physio - Chemical Methods (Instrumental Methods)

III. Microbiological Procedures

IV. Biological Procedures

I. Chemical Methods

a. Titrimetric Analysis

The analysis based on the fact that in all balanced chemical reactions utilized for the purpose. Equivalent weight of one substance reacts quantitatively with the equivalent weight of the other substance. The difference types of titration are as follows

Acid base titrations (neutralization titrations)

Non- aqueous titrations

Redox titrations (redox = oxidation - reduction)

Precipitation titrations

Complexometric titrations

b. Gravimetric Analysis

This method involves the conversion of the element or a radical to be determined into a pure stable compound readily convertible into a form suitable for weighing.

c. Gasometric Analysis

This type of analysis involves the measurement of the volume of gases. The volume of a gas set free in a given chemical reaction under the conditions similar to those described in the process. It may be noted that the volume of gas is taken at normal conditions and pressure or standard temperature and pressure (NTP/ STP) which is a temperature of 0^0C (273.09^o K) and the pressure of a column of 760mm/ Hg at 0^0C. If the reaction is taken place under different temperature and pressure, the volume is adjusted to standard conditions. A decrease in the volume of gas when a suitable reagent is placed to absorb one of the gases present. This decrease in volume is also reduced to STP.

The gases cyclopropane, carbon di oxide, nitrogen oxide, oxygen, octyl nitrite, nitrogen, amyl nitrite, ethylene and helium are determined by gasometric analysis. The measurement of volume of gases is usually done by means of gas burettes or nitro meters.

II. Physio - Chemical Methods (Instrumental Methods)

Initially analytical methods were depending on extraction procedure, volumetric and gravimetric methods. All these methods are nearly replaced by advanced instrumental methods. These methods are more sensitive, specific and accurate but cost factors of the instruments and their maintenance are the main draw backs. Various instrumental methods are classified depending on the property analyzed. The following table shows different instrumental methods with basic principles.

S. N0.	MEHTOD	BASIC PRINCIPLE
A	ELECTROANALYTICAL METHODS	
1	Potentiometry	Concerned with change in electrical properties of the system measures the change in electrode potential during a chemical reaction of the system
2	Conductometry	Measures the change in electrical conductivity during a chemical reaction
3	Polarography	Measure the current at various applied potential indicating the polarization at indicator electrode
4	Amperometry	Measure the change (or decrease) in current at a fixed potential during addition of titrant
B	SPECTROSCOPIC METHODS	
1	Absorption Spectroscopy (Ultraviolet-Visible and Infrared)	Measure the absorbance or percent transmittance during the interaction of monochromatic radiation (or particular wavelength) by the same
2	Fluorimetry	Measure the intensity of fluorescence caused by emission of electromagnetic radiation due to absorption of UV radiation
3	Flame Photometry	Measure the intensity of emitted light of particular wave length emitted by particular element
4	Turbidimetry	Measure the turbidity of a system by passing light beam in a turbid media

S. N0.	MEHTOD	BASIC PRINCIPLE
5	Nephlometry	Measure the opalescence of the medium by reflection of light by a colloidal solution
6	Atomic Absorption Spectrometry	Measure the intensity of absorption when atoms absorbs the monochromatic radiation
7	X-Ray Spectroscopy	Measure the position and intensity of spectral lines during emission of X ray spectrum by atoms under influence of X rays
8	Refractometry	Measure the refractive index by causing refraction of light by matter
9	Polarimetry	Measure optical reaction by causing the rotation of plane polarized light
10	Mass Spectroscopy	Observe the position and intensity of signals in mass spectrum by causing the ionization of molecules
11	NMR Spectroscopy	Observe the position and intensity lines in NMR spectrum when proton interact with electromagnetic radiation in radio frequency region
12	Thermal Methods	Measure the physical parameters of the system as a function of temperature. It includes thermo gravimetry, derivative gravimetry, differential thermal analysis
13	Radiometric Methods	Measure the radioactivity either present naturally or induced artificially

III. Microbiological Methods

In a microbiological assay, a comparison of inhibition of the growth of bacteria by a measured concentration of the antibiotic, which is to be examined, is made with that produced by known concentration of the standard preparation of an antibiotic having known activity.

IV. Biological Methods

When the potency of a drug or its derivative cannot be properly determined by physical or chemical methods and where it is possible to observe the biological effects of the drug on some type of living matter. The biological assays are carried out. The basis of such assay is to determine how much of the sample gives the same biological effect as a given quantity of the standard preparation. The sample and standard preparation are tested under identical conditions in all respect. In a typical bio – assay, a stimulus is applied to a subject is referred to as the dose and is indicated by a weight or in terms of the concentration of the preparation. The application of stimulus on a subject produces some observable effect and this is called the response. The response may be measured by the total weight or weight of some organ of the subject, blood sugar concentration, and diameter of inhibition zone or by some other physiological symptoms.

1.2 ULTRAVIOLET SPECTROSCOPY (Beckett and Stenlake, 2002)

This technique of ultra violet spectroscopy is one of most frequently employed method in pharmaceutical analysis. It involves the measurement of the amount of UV radiation (190 - 380 nm) or visible (380 - 800 nm) radiation absorbed by a substance in solution. Ultraviolet spectroscopy involves the promotion of electrons (σ, π, n electrons) from the ground state to higher energy state. It is useful to measure the number of conjugated double bonds and also aromatic conjugation with the various molecules.

The ultraviolet region of the electromagnetic spectrum is frequently subdivided into as follows:

- Far vacuum Ultraviolet region (10 - 200 nm)
- Near ultraviolet region (200 - 400 nm)
- Visible region (380 - 780 nm)

Origin and theory of ultraviolet spectra (Gurdeep R. Chatwal, et al., 2000)

Ultraviolet absorption spectra arise from transition of electron (or) electrons with in a molecule or anion from a lower to a higher electron energy level and the ultraviolet emission spectra arise from the reverse type of transmission. For radiation to cause electronic excitation it must be in the UV region of the electromagnetic spectrum.

Energy absorbed in the ultraviolet region produces change in the electronic energy of the molecule resulting from transition of valence electrons in the molecule. Three distinct types of electrons are involved in organic molecule. These are as follows

σ – Electrons

Theses electrons are involved in saturated bonds, such as those between carbons and hydrogen in paraffins. These bonds are known as σ bonds. As the amount of energy required to excite electron in σ bonds is much more than that produced by UV light, compounds containing σ bonds do not absorb UV radiation. These electrons do not absorb near UV radiation but absorb at vacuum UV radiation.

π – Electrons

These electrons are involved in unsaturated hydrocarbons. Typical compounds with π bonds are trienes and aromatic compounds.

n– Electrons

These electrons are non bonded electrons which are not involved in any bonding between atoms in molecules. (eg.) S, O, N and halogens.

Types of Electronic Transitions (Y.R. Sharma, 2009)

A molecule is excited by the absorption of energy (UV or Visible light). Its electrons are promoted from a bonding to an anti bonding orbital.

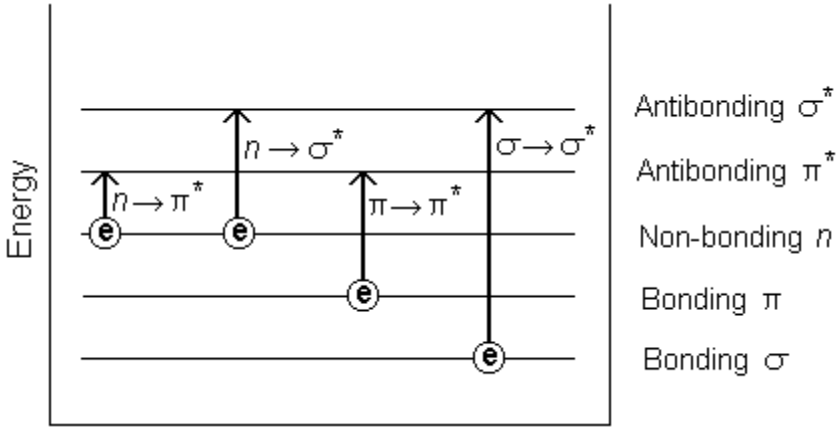
i. The anti bonding orbital which is associated with the excitation of σ – electrons is called σ* anti bonding orbital. It is represented as σ $\longrightarrow$ σ* transition.

ii. When a non - bonding electron gets promoted to an antibonding sigma orbital (σ*) then it represents n - σ* transition.

iii. Similarly π $\longrightarrow$ π* transition represents the promotion of π electrons to an anti bonding π* orbital.

iv. When n - electron (non - bonding) is promoted to anti bonding π orbital. It represents n $\longrightarrow$ π* transition.

v.



Electronic excitation energies

1.2.1 Visible Spectrophotometry (Mendham et al. 2002, B.K. Sharma, 2006)

Visible spectrophotometry involves the measurement of the amount of visible radiation (400 - 800 nm) absorbed by a colour solution. Using this, the quality of an element present is estimated from the intensity of the colour of the solution due to the presence of a coloured compound of that element. The more intense colour is the higher concentration of the element in solution.

Some compounds are self coloured and for other it is necessary to develop colour by the addition of one or more colour forming reagents (chromogenic reagents). The absorbing capacity of a coloured system is directly proportional to the amount of desired constituent.

Colorimetric analysis should satisfy following criteria.

- The colour reaction should be specific
- Proportionality change between colour and concentration
- Colour should be stable to permit an accurate reading
- Reproducible result should be notified
- Solution must be free from precipitate
- The colour reaction should be highly sensitive

In physics colorimetry refers to the measurement of colour and the determination involves neither the nature of the colourant nor its amounts. In chemistry, a general term called absorptiometry is used for chemical analysis through measurement of radiation.

Chemical systems which exhibit a selective light absorptive capacity or coloured, and hence the terms colorimetric analysis and colorimetry are often applied to the measurements of such system when the object is to determine the concentration of the consistutents responsible for the colour. In other words the variation of some component forms the basis of colorimetric analysis and it is concerned with the determination of the concentration of a substance by measurement of the relative absorption of light with respective of a known concentration of substance.

Colorimetric analysis is of two types:

Visual Colorimetry

This is the type of analysis in which natural or artificial white light is used as a light source and determinations are usually made with a colorimetry or colour comparator.

Photo Electric Colorimetry

In this type of analysis, eye is replaced by photo electric cell. The latter eliminates the error due to the personal characteristics of the observer. Photo electric colorimetry is usually employed with light contained within a comparatively narrow range of wavelengths obtained by passing white light through filters. The most important advantage of the colorimetric and spectrophotometric analysis is that they provide a significant method of determining minute quantities of substances. Some compounds are self coloured and other compounds are not. It is usually necessary to develop a colour by the addition of one or more colour forming agents. Chemical methods used to prepare suitable coloured solutions are usually called chromogenic reactions. The colour forming reagents are known as chromogenic reagents. The preparation of the coloured substance is as important as the measurement step in the visible spectrometry and hence in order to get good results a careful attention is extremely important while preparing a coloured solution.

The absorption capacity of a coloured system is directly proportional to the amount of the desired constituent; however the effect of other substance present in sample must known and the optimum condition under which the chromogenic reagent reacts with desired constituent.

The following are the most important prosperities of a coloured system which are suitable for measurement.

Sensitivity

The solution should be intensely coloured. Hence an easily detectable change in intensity must be obtained by small changes in the concentration. In this manner very small amount of the constituents can be determined.

Specificity

Only the desired constituent or some definitely related to it should develop a colour.

Stability

In order to make reliable measurement the intensity of the coloured solution should remain constant for long period.

Reproducibility

The hue as well as the intensity of the coloured solution must reproducible. The effect of the order of addition of reagents, the pH and other solution variables should be directly delineated.

Conformity to Beer's law

The measurement may be facilitated both for single and poly component system if Beers law is obeyed by the coloured solution. If Beers law does not confirm, adequate precautions are necessary.

The absorption laws (Y.R. Sharma, 2009)

There are two laws which govern the absorption of light by the molecules. These are,

(1) Lambert's Law

(2) Beer's Law

Lambert's Law

When a beam of monochromatic radiation passes through a homogenous absorbing medium, the rate of decrease of intensity of radiation with thickness of absorbing medium is proportional to the intensity of incident radiation.

$$I = I_0 e^{-kt}$$

Where, I_0 = Intensity of incident light

I = Intensity of emerged light

t = Thickness of the medium

Beer's Law

When a beam of monochromatic radiation is passed through a solution of an absorbing substance, the rate of decrease of intensity of radiation with concentration of the absorbing solution is directly proportional to the intensity of incident radiation.

$$I = I_0 e^{-kc}$$

Where, I_0 = intensity of incident light

I = Intensity of emerged light

c = concentration of the absorbing species

From these laws, the following empirical expression of Beer - Lambert's Law was constructed

$$\text{Log } (I_0/I_T) = \epsilon ct = A$$

Where, A= Absorbance or optical density or extinction

ϵ = Molar extinction co-efficient

c = Concentration of drug

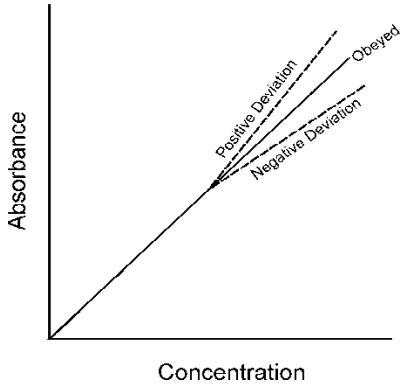
t = Path length

Limitations of Beer Lambert's Law

1. When different forms of the absorbing molecules are in equilibrium as in keto-enol tautomers.
2. When fluorescence compounds are present.
3. When solute and solvent forms complex through some sorts of association.

Deviations from Beer's Law (Gurdeep R. Chatwal, et al., 2000)

According to Beer's law, a straight line passing through the origin should be obtained, when a graph is plotted between absorbance and concentration. But there is always a deviation from linear relationship between absorbance and concentration and intact the shape of an absorption curve usually changes with changes in concentration of solution and unless precautions are observed. Deviations from the law may be positive or negative according to whether the resulting curve is concave upward or concave downward.



The latter two are generally known as instrumental deviation and chemical deviation.

Instrumental deviations

Stray radiation, improper slit width, fluctuation in single beam.

Chemical deviations

Hydrolysis, Association, Polymerization, Ionization, Hydrogen bonding.

Deviations from Beer's Law can arise due to the following factors

1. Beer's law will hold over a wide range of concentration provided the structure of coloured ion or of the coloured non electrolyte in the dissolved state does not change with concentration. If a coloured solution is having a foreign substance whose ions do not react chemically with the coloured components, its small concentration does not affect the light absorption and may also alter the value of extinction co - efficient.
2. Deviations may also occur if the coloured solute ions dissociates or associates.
3. Deviations may also occur due to the presence of impurities that fluorescence or absorb at absorption wavelength.
4. Deviations may occur if monochromatic light is not used.
5. Deviations may occur if the width of slit is not proper and therefore it allows undesirable radiations to fall on the detector.
6. Deviations may occur if the solution undergoes polymerization.
7. Beer's law cannot apply to suspensions but the latter can estimated calorimetrically after preparing a reference curve with known concentrations.

Choice of solvent (Gurdeep R. Chatwal, et al., 2000)

A suitable solvent for ultraviolet spectroscopy should meet the following requirements.

(i) It should not itself absorb radiations in the region under investigation.

(ii) It should be less polar so that it has minimum interaction with the solute molecule.

The most commonly employed solvent is 95% ethanol. It is cheap, has good dissolving power and does not absorb radiation above 210 nm. In other words it is

transparent above 210 nm. Commercial ethanol should not be used as it contains some benzene which undergoes absorption in the UV range at about 280. Some other solvents which are transparent above 210 nm are n-hexane, cyclohexane, methanol, water and ether, benzene, chloroform, and carbon tetrachloride cannot be used because they absorb in the range of about 240 - 280 nm.

Hexane and other hydrocarbon are sometimes preferred to polar solvents because they have minimum interaction with the solute molecules.

Solvent Effects

The position and the intensity of absorption maximum is shifted for a particular chromophore by changing the polarity of the solvent. By increasing the polarity of solvent, compounds like dienes and conjugated hydrocarbons do not experience any appreciable shift. The absorption maximum for the polar compounds is usually shifted with the change in polarity of the solvents. α and β unsaturated carbonyl compounds show two different shifts.

a) n $\longrightarrow$ π* transition

The absorption band moves to short wavelength by increasing the polarity of the solvent. In n $\longrightarrow$ π* transition the ground state is more polar as compared to the excited state. The hydrogen bonding with solvent molecules take place to lesser extent with the carbonyl group in the excited state.

b) π $\longrightarrow$ π* transition

The absorption band moves to longer wavelength by increasing the polarity of the solvent. The dipole interactions with the solvent molecules lower the energy of the excited state more than that of the ground state.

Chromophores and Auxochromes (Jagmohan, 2005)

A. Chromophores

"The presence of one or more unsaturated groups responsible for electronic absorption is called as chromophores".

e.g. C = C, C ≡ C, C = N, C ≡ N, C = O

B. Auxochromes

An auxochrome is an auxiliary group which interacts with the chromophore causing a bathochromic shift. The presence of auxochrome causes a shift in the UV or Visible absorption maximum to the longer wavelength.

e.g. NH_2, NR_2, OH, OR, SH, NHR.

1.2.1.1 Instrumentation (Gurdeep R. Chatwal, et al., 2000)

All photometers, colorimeters and spectrophotometers have the following basic components

i) Radiation Source

For UV region

Hydrogen discharge lamp, Deuterium discharge lamp, Xenon arc Lamp.

For Visible region

The tungsten halogen lamp is the most common source of visible radiation.

ii) Filters and Monochromatoros

The filters and monochromators are used to disperse the radiation according to the wavelength. The essential of a monochromator are an entrance slit, a dispersing element and an exit slit. The entrance slit sharply defines the incoming beam of heterochromatic radiation. The dispersing element disperses the heterochromatic radiation into its component wavelengths where as exit slit allows the nominal

wavelength together with a band of wavelength on either side of it. The position of the dispersing element is always adjusted by rotating it to vary the nominal wavelength passing through the exit slit.

For UV region

The dispersing element may be a prism or grating. The prisms are generally made of glass, quartz or fused silica. Glass has the highest resolving power but it is not transparent to radiation having the wavelength between 2000 and 3000 A because glass absorbs strongly in this region. Quartz and fused silica prism which are transparent throughout the entire UV range are widely used in UV spectrophotometers.

Types of monochromators

1) Prisms

2) Gratings

1) Prisms are of two types

i) Refractive prism

ii) Reflective prism

2) Gratings are of two types

i) Diffraction grating

ii) Transmission grating

For Visible region

Filters or monochromators or both are used

Filters

A light filter is a device that allows light of the required wavelength to pass but absorbs light of other wavelengths wholly or partially. Thus, a suitable filter can be select a desired wavelength band. It means that a particular filter may be used for a specific analysis. If analysis is carried out for several species, a large number of filters have to be used and interchanged. This method is very useful for routine analysis.

Types of filters

Filters are two types,

i) Absorption filters

ii) Interference filters

iii) Sample cell

These are containers for the sample and reference solutions and must be transparent to the radiation passing through.

UV region

The cells made up of quartz.

Visible region

The cells made of glass.

iv) Detectors

Detectors used in UV/Visible spectrophotometers can be called as photometric detectors. In these detectors the light energy is converted to electrical signal which can be recorded. The types of detectors used are

Barrier Layer cell (or) Photo Voltaic cell.

Photo tubes (or) Photo emissive tubes

Photomultiplier tubes

Photo diode

1.2.2 Difference Spectrophotometry (Beckett and Stenlake, 2002)

The selectivity, accuracy of spectrophotometric analysis of samples containing absorbing interferents may be markedly improved by the technique of difference spectrophotometric assay is that the measured value is the difference absorbance (A) between two equimolar solutions of the analyte in different chemical forms which exhibits difference spectral characteristics. The criteria for applying difference spectrophotometry to the assay of a substance in the presence of other absorbing substances are that

- Reproducible changes may be induced in the spectrum of analyte by the addition of one or more reagents.
- The absorbance of the interfering substance is not altered by the reagents.

The simplest and most commonly employed technique for altering the spectral properties of the analyte is the adjustment of the pH by means of aqueous solutions of acid, alkali or buffers. The ultra violet visible absorption spectra of many substances containing ionisable functional groups, e.g. phenols, aromatic carboxylic acids and amines, are dependent on the state of ionization of the functional groups and consequerently on the pH of the solution.

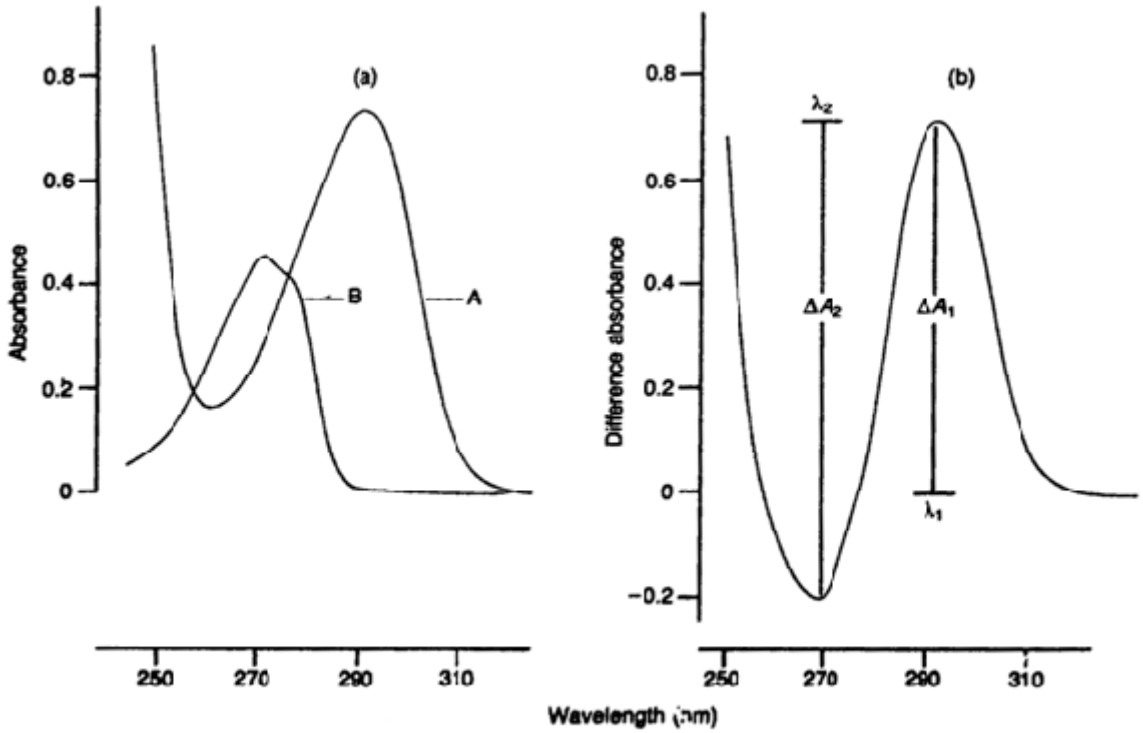


Fig. 7.7(a) The absorption spectra of equimolar solutions of phenylephrine hydrochloride (50 μg ml^{-1}) in (A) 0.1M sodium hydroxide and (B) 0.1M hydrochloric acid. (b) The difference absorption spectrum of solution B relative to solution A.

The measured value in a quantitative difference spectrophotometric assay is the ΔA at any suitable wavelength measured to the baseline, e.g. ΔA_1 at λ_1 or amplitude between an adjacent maximum and minimum, e.g: ΔA_2 at λ_2 and λ_1

$$\Delta A = A_{alk} - A_{acid}$$

Where, A_{alk} and A_{acid} are the individual absorbances at λ_1 in 0.1M sodium hydroxide and 0.1M hydrochloric acid solution, respectively.

$$\Delta A = \Delta\ abc$$

1.2.3 Quantitative Spectrophtometric Methods (Beckett and Stenlake, 2002)

The assay of an absorbing substance may be quickly carried out by preparing a solution in a transparent solvent and measuring its absorbance at a suitable wavelength. The wavelength normally selected is a wavelength of maximum absorption (λ_{max}),

where small errors in setting the wavelength scale have little effects on the measured absorbance.

a. Assay of substances in single component samples

Absorption spectroscopy is one of the most useful tools available to the chemist for quantitative analysis. The most important characteristics of photometer and spectrophotometric method are high selectivity and ease of convenience. Quantitative analysis (assay of an absorbing substance) can be done using following methods.

- Use of $A_{1\,cm}^{1\%}$ values
- Use of calibration graph (multiple standard method)
- By single or double point standardization method.

i) Use of $A_{1\,cm}^{1\%}$ values

This method can be used for estimation of drug from formulations or raw material, when reference standard not available. The use of standard value $A_{1\,cm}^{1\%}$ avoids the need to prepare a standard solution of the reference substance in order to determine its absorptivity, and is of advantage in situations where it is difficult or expensive to obtain a sample of the reference substance.

ii. Use of calibration graph

In this procedure the absorbances of a number (typically 4-6) of standard solutions of the reference substance at concentrations encompassing the sample concentrations are measured and a calibration graph is constructed. The concentration of the analyte in the sample solution is read from the graph as the concentration corresponding to the absorbance of the solution. Calibration data are essential if the absorbance has a non-linear relationship with concentration, or if the absorbance or linearity is dependent on the assay conditions. In certain visible spectrophotometric

assays of colorless substances, based upon conversion to coloured derivatives by heating the substance with one or more reagents, slight variation of assay conditions, e.g. P^H, temperature and time of heating, may rise to a significant variation of absorbance, and experimentally derived calibration data are required for each set of samples.

iii. Single or double point standardization

The single point procedure involves the measurement of the absorbance of a sample solution and of a standard solution of the reference substance. The standard and the sample solution are prepared in similar manner; ideally the concentration of the standard solution should be close to that of the sample solution. The concentration of the substance in the sample is calculated using following formula.

$$\mathbf{C_{test} = A_{test} \times C_{std} / A_{std}}$$

Where,

C_{test} and C_{std} are the concentration in the sample and standard solutions respectively.

A_{test} and A_{std} are the absorbance of the sample and standard solutions respectively.

In double point standardization, the concentration of one of the standard solution is greater than that of the sample while the other standard solution has a lower concentration than the sample. The concentration of the substance in the sample solution is given by

$$\mathbf{C_{test} = \frac{(A_{test} - A_{std1})(C_{std1}-C_{std2}) + C_{std1}(A_{std1}-A_{std2})}{A_{std1}-A_{std2}}}$$

Where,

C_{std} is the concentration of the standard solution.

A_{test} and A_{std} are the absorbance of the sample and standard solution respectively.

Std_1 and Std_2 are the more concentrated standard and less concentrated standard respectively.

1.3 HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY

(Sethi, P.D., 1996, Sethi, P.D., Dilip Chareganokar.1999)

HPTLC is a most versatile and simple technique for both qualitative and quantitative analysis of drugs in the pure state, to those extracted from pharmaceutical formulations and to biological samples. This technique is the advancement of the thin layer chromatography. It involves the same principle of thin layer chromatography but differs from thin layer chromatography in parameters such as speed, accuracy and cost of analysis. Unlike other chromatographic techniques, HPTLC does not require more space or bulk instrument or any special training for the handling of instruments.

1.3.1 Steps Involved

The various steps involved in the process of HPTLC are selection of plates, sample preparation, pre-washing of plates, activation of plates, application of the sample, development and detection.

1.3.1.1 Selection of plates

The plates used in HPTLC are either handmade plates or pre-coated plates. The handmade plates differ from the pre-coated plates in the support material. The thickness of the plates varies from 100 to 250 μm. The sorbent used in the plates are cellulose,

cellulose with starch, microcrystalline cellulose, silica gel, silica gel G, silica gel with starch and acetylated cellulose

The handmade plates are made with a glass support whereas; the pre-coated plates are made with supports such as polyester sheets and aluminum sheets.

1.3.1.2 Pre-washing

The pre-washing is done to remove any impurity that might have adhered to the plate surface. The adsorbents coated over the plate have the tendency to absorb the atmospheric moisture and other impurities suspended in the air. Thus pre-washing prevents any interference of the impurity during the development of the chromatogram.

The pre-washing is done by ascending, dipping or by continuous mode. Methanol is the commonly used solvent for the washing of plates. The solvents such as methanol in chloroform (1:1), ethyl acetate in methanol (1:1), chloroform: methanol: ammonia (90:10:1) are also used in the pre-washing of the plates

1.3.1.3 Activation of plates

The plates are activated by placing it in a oven at 110 to 120º C for 30 minutes. Activation removes any moisture which maybe physically absorbed to the surface of the plate.

1.3.1.4 Application of sample

The application of sample is done using a device. The improper application may lead to improper resolution of the samples. While applying the sample, care must be taken so that the layer of the adsorbent is not damaged. The sample spots are made as bands for better separation.

1.3.1.5 Mobile phase

The solvents used in the preparation of mobile phase must be of good quality as poor grade of solvents decreases the resolution of the sample. The components of the

mobile phase are measured separately and then are mixed in a vessel and then introduced into the developing chamber. The mobile phase should not react with the analyte or the stationary phase used.

1.3.1.6 Chamber saturation

Chamber saturation is done by allowing the mobile phase to completely saturate inside the closed chamber only with mobile phase. The chamber saturation helps in faster development of the chromatogram. Unsaturated chamber gives improper Rf values due to the evaporation of the solvents at the solvent front.

1.3.1.7 Development and detection

After the chamber saturation the plates are developed using any one of the various methods available. The development techniques are ascending, descending, two dimensional, horizontal, radial etc. The tanks or the chambers used in the development process are twin trough chambers, rectangular chambers, circular chambers and automated multiple development chambers.

After the development the plates are removed and dried to evaporate the mobile phase. The detection is made by using UV, visible light and by fluorescence.

Derivatizations are required for those samples which do not respond to UV light or does not have fluorescence. Densitometry is in situ measurement of visible light, UV radiations and fluorescence on the plate.

1.3.2 Factors Influencing HPTLC

The resolution and separation of spots in a HPTLC system are influenced by various parameters such as type of stationary phase, thickness of adsorbent, mobile phase, developing chamber, size of the sample spot, mode of development and relative humidity.

1.4 ANALYTICAL METHOD VALIDATION

(Code Q2A; Q2B; ICH Guidelines, U.S.P)

1.4.1 Introduction

This document presents a discussion of the characteristics for consideration during the validation of the analytical procedures included as part of registration applications submitted within the EC, Japan and USA. This document does not necessarily seek to cover the testing that may be required for registration in, or export to, other areas of the world. Furthermore, this text presentation serves as a collection of terms, and their definitions, and is not intended to provide direction on how to accomplish validation. These terms and definitions are meant to bridge the differences that often exist between various compendia and regulators of the EC, Japan and USA.

The objective of validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose. A tabular summation of the characteristics applicable to identification, control of impurities and assay procedures is included. Other analytical procedures may be considered in future additions to this document.

Types of Analytical Procedures to be Validated

The discussion of the validation of analytical procedures is directed to the four most common types of analytical procedures. They are identification tests, quantitative tests for impurities content, limit tests for the control of impurities and quantitative tests of the active moiety in samples of drug substance or drug product or other selected component(s) in the drug product.

Although there are many other analytical procedures, such as dissolution testing for drug products or particle size determination for drug substance, these have not been addressed in the initial text on validation of analytical procedures. Validation

of these additional analytical procedures are equally important to those listed herein and may be addressed in subsequent documents.

A brief description of the types of tests considered in this document is provided below.

- Identification tests are intended to ensure the identity of an analyte in a sample. This is normally achieved by comparison of a property of the sample (e.g., spectrum, chromatographic behaviour, chemical reactivity, etc) to that of a reference standard.
- Testing for impurities can be either a quantitative test or a limit test for the impurity in a sample. Either test is intended to accurately reflect the purity characteristics of the sample. Different validation characteristics are required for a quantitative test than for a limit test.
- Assay procedures are intended to measure the analyte present in a given sample. In the context of this document, the assay represents a quantitative measurement of the major component(s) in the drug substance. For the drug product, similar validation characteristics also apply when assaying for the active or other selected component(s). The same validation characteristics may also apply to assays associated with other analytical procedures (e.g., dissolution).

The objective of the analytical procedure should be clearly understood since this will govern the validation characteristics which need to be evaluated. Typical validation characteristics which should be considered are listed below

Accuracy

Precision

Repeatability

Intermediate Precision

Specificity

Detection Limit

Quantitation Limit

Linearity

Range

Each of these validation characteristics is defined in the attached Glossary. The table lists those validation characteristics regarded as the most important for the validation of different types of analytical procedures. This list should be considered typical for the analytical procedures cited but occasional exceptions should be dealt with on a case-by-case basis. It should be noted that robustness is not listed in the table but should be considered at an appropriate stage in the development of the analytical procedure.

Furthermore revalidation may be necessary in the following circumstances

- Changes in the synthesis of the drug substance
- Changes in the composition of the finished product
- Changes in the analytical procedure.

The degree of revalidation required depends on the nature of the changes. Certain other changes may require validation as well.

EVALUATION SIGNIFICANCE TABLE

Type of analytical procedures characteristics	IDENTIFICATION	TESTING FOR IMPURITIES Quantitation	 limit	ASSAY - dissolution (measurement only) - content/potency
Accuracy	-	+	-	+
Precision				
Repeatability	-	+	-	+
Intermediate Precision	-	+ (1)	-	+ (1)
Specificity (2)	+	+	+	+
Detection Limit	-	- (3)	+	-
Quantitation Limit	-	+	-	-
Linearity	-	+	-	+
Range	-	+	-	+

- signifies that this characteristic is not normally evaluated

+ signifies that this characteristic is normally evaluated

(1) In cases where reproducibility (see glossary) has been performed, intermediate precision is not needed

(2) Lack of specificity of one analytical procedure could be compensated by other supporting analytical procedure(s)

(3) May be needed in some cases

1.4.2 GLOSSARY

ANALYTICAL PROCEDURE

The analytical procedure refers to the way of performing the analysis. It should describe in detail the steps necessary to perform each analytical test. This may include but is not limited to: the sample, the reference standard and the reagents preparations, use of the apparatus, generation of the calibration curve, use of the formulae for the calculation, etc.

SPECIFICITY

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc. Lack of specificity of an individual analytical procedure may be compensated by other supporting analytical procedure(s).

This definition has the following implications:

Identification: to ensure the identity of an analyte.

Purity Tests: to ensure that all the analytical procedures performed allow an accurate statement of the content of impurities of an analyte, i.e. related substances test, heavy metals, residual solvents content, etc.

Assay (content or potency):

To provide an exact result this allows an accurate statement on the content or potency of the analyte in a sample.

ACCURACY

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. This is sometimes termed trueness.

PRECISION

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility.

Precision should be investigated using homogeneous, authentic samples. However, if it is not possible to obtain a homogeneous sample it may be investigated using artificially prepared samples or a sample solution.

The precision of an analytical procedure is usually expressed as the variance, standard deviation or coefficient of variation of a series of measurements.

REPEATABILITY

Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision.

INTERMEDIATE PRECISION

Intermediate precision expresses within-laboratories variations: different days, different analysts, different equipment, etc.

REPRODUCIBILITY

Reproducibility expresses the precision between laboratories (collaborative studies, usually applied to standardization of methodology).

DETECTION LIMIT

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value.

QUANTITATION LIMIT

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The quantitation limit is a parameter of quantitative assays for low levels of compounds in sample matrices, and is used particularly for the determination of impurities and/or degradation products.

LINEARITY

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample.

RANGE

The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.

ROBUSTNESS

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

1.4.3 VALIDATION OF ANALYTICAL PROCEDURES

METHODOLOGY

INTRODUCTION

This document is complementary to the parent document which presents a discussion of the characteristics that should be considered during the validation of analytical procedures. Its purpose is to provide some guidance and recommendations on how to consider the various validation characteristics for each analytical procedure**.** In some cases (for example, demonstration of specificity), the overall capabilities of a number of analytical procedures in combination may be investigated in order to ensure the quality of the drug substance or drug product. In addition, the document provides an indication of the data which should be presented in a registration application.

All relevant data collected during validation and formulae used for calculating validation characteristics should be submitted and discussed as appropriate.

Approaches other than those set forth in this guideline may be applicable and acceptable. It is the responsibility of the applicant to choose the validation procedure and protocol most suitable for their product. However it is important to remember that the main objective of validation of an analytical procedure is to demonstrate that the procedure is suitable for its intended purpose. Due to their complex nature, analytical procedures for biological and biotechnological products in some cases may be approached differently than in this document.

Well-characterized reference materials, with documented purity, should be used throughout the validation study. The degree of purity necessary depends on the intended use.

In accordance with the parent document, and for the sake of clarity, this document considers the various validation characteristics in distinct sections. The arrangement of these sections reflects the process by which an analytical procedure may be developed and evaluated.

In practice, it is usually possible to design the experimental work such that the appropriate validation characteristics can be considered simultaneously to provide a sound, overall knowledge of the capabilities of the analytical procedure, for instance: specificity, linearity, range, accuracy and precision.

SPECIFICITY

An investigation of specificity should be conducted during the validation of identification tests, the determination of impurities and the assay. The procedures used to demonstrate specificity will depend on the intended objective of the analytical procedure.

It is not always possible to demonstrate that an analytical procedure is specific for a particular analyte (complete discrimination). In this case a combination of two or more analytical procedures is recommended to achieve the necessary level of discrimination.

Identification

Suitable identification tests should be able to discriminate between compounds of closely related structures which are likely to be present. The discrimination of a procedure may be confirmed by obtaining positive results (perhaps by comparison with a known reference material) from samples containing the analyte, coupled with negative results from samples which do not contain the analyte. In addition, the identification test may be applied to materials structurally similar to or closely related to the analyte to

confirm that a positive response is not obtained. The choice of such potentially interfering materials should be based on sound scientific judgement with a consideration of the interferences that could occur.

Assay and Impurity Test (s)

For chromatographic procedures, representative chromatograms should be used to demonstrate specificity and individual components should be appropriately labelled. Similar considerations should be given to other separation techniques.

Critical separations in chromatography should be investigated at an appropriate level. For critical separations, specificity can be demonstrated by the resolution of the two components which elute closest to each other.

In cases where a non-specific assay is used, other supporting analytical procedures should be used to demonstrate overall specificity. For example, where a titration is adopted to assay the drug substance for release, the combination of the assay and a suitable test for impurities can be used. The approach is similar for both assay and impurity tests

Impurities are available

For the assay, this should involve demonstration of the discrimination of the analyte in the presence of impurities and/or excipients; practically, this can be done by spiking pure substances (drug substance or drug product) with appropriate levels of impurities and/or excipients and demonstrating that the assay result is unaffected by the presence of these materials (by comparison with the assay result obtained on unspiked samples).

For the impurity test, the discrimination may be established by spiking drug substance or drug product with appropriate levels of impurities and demonstrating the separation of these impurities individually and/or from other components in the sample matrix.

Impurities are not available

If impurity or degradation product standards are unavailable, specificity may be demonstrated by comparing the test results of samples containing impurities or degradation products to a second well-characterized procedure e.g. pharmacopoeial method or other validated analytical procedure (independent procedure). As appropriate, this should include samples stored under relevant stress conditions: light, heat, humidity, acid/base hydrolysis and oxidation.

- For the assay, the two results should be compared;
- For the impurity tests, the impurity profiles should be compared.

Peak purity tests may be useful to show that the analyte chromatographic peak is not attributable to more than one component (e.g., diode array, mass spectrometry).

LINEARITY

A linear relationship should be evaluated across the range (see section 3) of the analytical procedure. It may be demonstrated directly on the drug substance (by dilution of a standard stock solution) and/or separate weighing of synthetic mixtures of the drug product components, using the proposed procedure. The latter aspect can be studied during investigation of the range.

Linearity should be evaluated by visual inspection of a plot of signals as a function of analyte concentration or content. If there is a linear relationship, test results

should be evaluated by appropriate statistical methods, for example, by calculation of a regression line by the method of least squares. In some cases, to obtain linearity between assays and sample concentrations, the test data may need to be subjected to a mathematical transformation prior to the regression analysis. Data from the regression line itself may be helpful to provide mathematical estimates of the degree of linearity.

The correlation coefficient, y-intercept, slope of the regression line and residual sum of squares should be submitted. A plot of the data should be included. In addition, an analysis of the deviation of the actual data points from the regression line may also be helpful for evaluating linearity.

Some analytical procedures, such as immunoassays, do not demonstrate linearity after any transformation. In this case, the analytical response should be described by an appropriate function of the concentration (amount) of an analyte in a sample.

For the establishment of linearity, a minimum of 5 concentrations is recommended. Other approaches should be justified.

RANGE

The specified range is normally derived from linearity studies and depends on the intended application of the procedure. It is established by confirming that the analytical procedure provides an acceptable degree of linearity, accuracy and precision when applied to samples containing amounts of analyte within or at the extremes of the specified range of the analytical procedure. The following minimum specified ranges should be considered:

- for the assay of a drug substance or a finished (drug) product: normally from 80 to 120 percent of the test concentration;

- for content uniformity, covering a minimum of 70 to 130 percent of the test concentration, unless a wider more appropriate range, based on the nature of the dosage form (e.g., metered dose inhalers), is justified;

- For dissolution testing: +/-20 % over the specified range;

e.g., if the specifications for a controlled released product cover a region from 20%, after 1 hour, up to 90%, after 24 hours, the validated range would be 0-110% of the label claim.

- For the determination of an impurity: from the reporting level of an impurity to 120% of the specification; for impurities known to be unusually potent or to produce toxic or unexpected pharmacological effects, the detection/quantitation limit should be commensurate with the level at which the impurities must be controlled;

Note: for validation of impurity test procedures carried out during development, it may be necessary to consider the range around a suggested (probable) limit.

- if assay and purity are performed together as one test and only a 100% standard is used, linearity should cover the range from the reporting level of the impurities to 120% of the assay specification.

ACCURACY

Accuracy should be established across the specified range of the analytical procedure.

Assay

Drug Substance

Several methods of determining accuracy are available

a) Application of an analytical procedure to an analyte of known purity (e.g. reference material)

b) Comparison of the results of the proposed analytical procedure with those of a second well-characterized procedure, the accuracy of which is stated and/or defined (independent procedure, see 1.2.)

c) Accuracy may be inferred once precision, linearity and specificity have been established.

Drug Product

Several methods for determining accuracy are available

a) Application of the analytical procedure to synthetic mixtures of the drug product components to which known quantities of the drug substance to be analysed have been added

b) in cases where it is impossible to obtain samples of all drug product components , it may be acceptable either to add known quantities of the analyte to the drug product or to compare the results obtained from a second, well characterized procedure, the accuracy of which is stated and/or defined (independent procedure, see 1.2.)

c) Accuracy may be inferred once precision, linearity and specificity have been established.

Impurities (Quantitation)

Accuracy should be assessed on samples (drug substance/drug product) spiked with known amounts of impurities.

In cases where it is impossible to obtain samples of certain impurities and/or degradation products, it is considered acceptable to compare results obtained by an independent procedure (see 1.2.). The response factor of the drug substance can be used.

It should be clear how the individual or total impurities are to be determined e.g., weight/weight or area percent, in all cases with respect to the major analyte.

Recommended Data

Accuracy should be assessed using a minimum of 9 determinations over a minimum of 3 concentration levels covering the specified range (e.g., 3 concentrations/ 3 replicates each of the total analytical procedure).

Accuracy should be reported as percent recovery by the assay of known added amount of analyte in the sample or as the difference between the mean and the accepted true value together with the confidence intervals.

PRECISION

Validation of tests for assay and for quantitative determination of impurities includes an investigation of precision.

Repeatability

Repeatability should be assessed using

a) a minimum of 9 determinations covering the specified range for the procedure (e.g., 3 concentrations/3 replicates each)

b) a minimum of 6 determinations at 100% of the test concentration.

Intermediate Precision

The extent to which intermediate precision should be established depends on the circumstances under which the procedure is intended to be used. The applicant should establish the effects of random events on the precision of the analytical procedure. Typical variations to be studied include days, analysts, equipment, etc. It is not

considered necessary to study these effects individually. The use of an experimental design (matrix) is encouraged.

Reproducibility

Reproducibility is assessed by means of an inter-laboratory trial. Reproducibility should be considered in case of the standardization of an analytical procedure, for instance, for inclusion of procedures in pharmacopoeias. These data are not part of the marketing authorization dossier.

Recommended Data

The standard deviation, relative standard deviation (coefficient of variation) and confidence interval should be reported for each type of precision investigated.

DETECTION LIMIT

Several approaches for determining the detection limit are possible, depending on whether the procedure is a non-instrumental or instrumental. Approaches other than those listed below may be acceptable.

Based on Visual Evaluation

Visual evaluation may be used for non-instrumental methods but may also be used with instrumental methods.

The detection limit is determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected.

Based on Signal-to-Noise

This approach can only be applied to analytical procedures which exhibit baseline noise. Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and establishing the minimum concentration at which the analyte can be reliably detected. A signal-to-noise ratio between 3 or 2:1 is generally considered acceptable for estimating the detection limit.

Based on the Standard Deviation of the Response and the Slope

The detection limit (DL) may be expressed as

$$DL = \frac{3.3\,\sigma}{S}$$

Where, σ = the standard deviation of the response

S = the slope of the calibration curve

The slope S may be estimated from the calibration curve of the analyte. The estimate of σ may be carried out in a variety of ways, for example

Based on the Standard Deviation of the Blank

Measurement of the magnitude of analytical background response is performed by analyzing an appropriate number of blank samples and calculating the standard deviation of these responses.

Based on the Calibration Curve

A specific calibration curve should be studied using samples containing an analyte in the range of DL. The residual standard deviation of a regression line or the standard deviation of y-intercepts of regression lines may be used as the standard deviation.

Recommended Data

The detection limit and the method used for determining the detection limit should be presented. If DL is determined based on visual evaluation or based on signal to noise ratio, the presentation of the relevant chromatograms is considered acceptable for justification.

In cases where an estimated value for the detection limit is obtained by calculation or extrapolation, this estimate may subsequently be validated by the independent analysis of a suitable number of samples known to be near or prepared at the detection limit.

QUANTITATION LIMIT

Several approaches for determining the quantitation limit are possible, depending on whether the procedure is a non-instrumental or instrumental. Approaches other than those listed below may be acceptable.

Based on Visual Evaluation

Visual evaluation may be used for non-instrumental methods but may also be used with instrumental methods.

The quantitation limit is generally determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be quantified with acceptable accuracy and precision.

Based on Signal-to-Noise

This approach can only be applied to analytical procedures that exhibit baseline noise.

Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those

of blank samples and by establishing the minimum concentration at which the analyte can be reliably quantified. A typical signal-to-noise ratio is 10:1.

Based on the Standard Deviation of the Response and the Slope

The quantitation limit (QL) may be expressed as:

$$QL = \frac{10\,\sigma}{S}$$

Where, σ = the standard deviation of the response

S = the slope of the calibration curve

The slope S may be estimated from the calibration curve of the analyte. The estimate of σ may be carried out in a variety of ways for example:

Based on Standard Deviation of the Blank

Measurement of the magnitude of analytical background response is performed by analyzing an appropriate number of blank samples and calculating the standard deviation of these responses.

Based on the Calibration Curve

A specific calibration curve should be studied using samples, containing an analyte in the range of QL. The residual standard deviation of a regression line or the standard deviation of y-intercepts of regression lines may be used as the standard deviation.

Recommended Data

The quantitation limit and the method used for determining the quantitation limit should be presented.

The limit should be subsequently validated by the analysis of a suitable number of samples known to be near or prepared at the quantitation limit.

ROBUSTNESS

The evaluation of robustness should be considered during the development phase and depends on the type of procedure under study. It should show the reliability of an analysis with respect to deliberate variations in method parameters.

If measurements are susceptible to variations in analytical conditions, the analytical conditions should be suitably controlled or a precautionary statement should be included in the procedure. One consequence of the evaluation of robustness should be that a series of system suitability parameters (e.g., resolution test) is established to ensure that the validity of the analytical procedure is maintained whenever used.

Examples of typical variations are

- Stability of analytical solutions;
- Extraction time.

In the case of liquid chromatography, examples of typical variations are

- Influence of variations of pH in a mobile phase,
- Influence of variations in mobile phase composition,
- Different columns (different lots and/or suppliers),
- Temperature,
- Flow rate.

In the case of gas-chromatography, examples of typical variations are

- Different columns (different lots and/or suppliers)
- Temperature
- Flow rate.

1.5 STATICAL PARAMETERS (Mendham et al. 2002)

Linear Regression

Once linear relationship has been shown to have a high probability by the value of the correlation coefficient 'r' then the best straight line through the data points has to be estimated. This can be often done by visual inspection of graph but in many cases it is far more sensible to evaluate the best straight line by linear regression.

The equation of straight line is

$$Y = mx + c$$

Where, y the dependent variable is plotted as result changing x the independent variable.

To obtain regression line 'y ' on 'x ' the slope 'm' of the line intercept 'c' on the y axis are given by the following formula.

$$m = \frac{N\sum xy - (\sum x)(\sum y)}{N\sum x^2 - (\sum x)^2}$$

$$c = \frac{(\sum y)(\sum x^2) - (\sum x)(\sum xy)}{N\sum x^2 - (\sum x)^2}$$

Correlation Coefficient

The correlation co-efficient is used as a measure of the correlation between two variables. When variables x and y are correlated rather than being functionally related. The person correlation co efficient is one of the most convenient to calculate. This is given by

$$r = \frac{n \sum x_1 y_1 - \sum x_1 y_1}{\{[n \sum x_1{}^2 - (\sum x_1{}^2][n \sum y_1{}^2 - (\sum y_1{}^2]\}^{1/2}}$$

Where n is the number of data points.

The maximum, value of r is 1. When this occurs, there is exact correlation between the two variables. When r is zero, there is complete independence of the variables. The minimum value of r is -1. A negative correlation co-efficient indicates that the assumed dependence is opposite to what exists and therefore a positive co-efficient for the revered relation. The fit must be quite poor before r become smaller than about 0.98 and is really very poor when less than 0.9.

Residual Sum of Square

This is a measure of the variability of the data around the line of best fit that has been defined by linear regression. If all the data fall exactly on the calculated line of best fit, this would imply that the experiment has no associated error and, accordingly, the Residual Sum of Squares is equal to zero, the Residual Sum of Squares is calculated with knowledge of the total and regression sum of squares as follows.

$$SS_{residual} = SS_{total} - SS_{regression}$$

Where,

$$SS_{total} = \sum(y - Y)^2 = \sum y2 - \sum X^2 - (\sum X)^2/n$$

$$SS_{regression} = \frac{[(\sum Xy) - (\sum X \sum y/n)]^2}{\sum x^2 - (\sum X)^2/n}$$

Standard Deviation

It is commonly used in statistics as a measure of precision and is more meaning full than is the average deviation. It may be thought of as a root mean square deviation of values from their average and is expressed mathematically as

Where

$$S = \sqrt{\frac{\sum_{i=1}^{i=n} (x - \bar{x})}{N-1}}$$

S = Standard deviation

If N is large (50 or more) then of course it of immaterial whether the term in the denomination is N - 1 or N.

Σ = sum

$\bar{x}$ = Mean or arithmetic average.

$x - \bar{x}$ = Deviation of a value from the mean

N = Number of observations

Percentage Relative Standard Deviation (% RSD)

It is also known as co efficient of variation CV. It is defined as the standard deviation (SD) expressed as the percentage of mean.

$$\text{RSD (\%)} = \frac{\text{S.D}}{\bar{x}} \times 100$$

Where,

SD = Standard deviation

The variance is defined as S^2 and is more important in statistics than S itself. However the latter is much more commonly used with chemical data.

Standard Error of Mean (SE)

The standard error of mean can be defined as the value obtained by the division of standard deviation by square root of number of observations. It is mathematically expressed as,

$$\text{SE.} = \frac{\text{S.D}}{\sqrt{\text{n}}}$$

Where,

SD = Standard deviation.

N = number of observations.

Confidence Interval (CI)

When a small number of observations are made, the value of the standard deviation **S** does not by itself give a measure of how close the sample mean $\bar{x}$ might be to the true mean. But it is possible to calculate a Confidence Interval to estimate the range with in which the true mean may be found. The limits of this Confidence Interval known as the Confidence Limits.

$$CI = \bar{x} - t_{\frac{\propto}{2},n+1}\frac{s}{\sqrt{n}}, \qquad \bar{x} + t_{\frac{\propto}{2},n+1}\frac{s}{\sqrt{n}}$$

Where,

T = Parameters that depends up on the number of degrees of freedom x

S = Average Standard Deviation

$\bar{x}$ = Average mean

n = Number of observations

Literature Review

2. LITERATURE REVIEW

2.1 DRUG PROFILE

(http://www.drugs.com/monograph/tapentadol-hydrochloride.html)

(http://www.chemspider.com/Chemical-Structure.8013742.html#pred)

(http://www.medlink.com/medlinkcontent.asp)

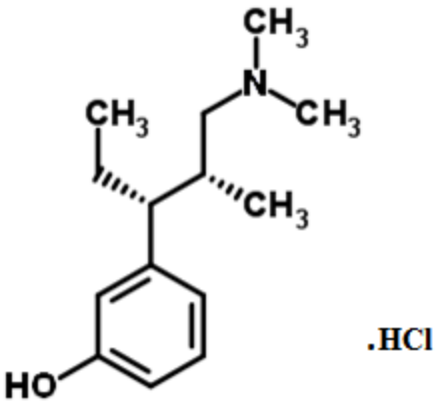
(http://www.druglib.com/druginfo/nucynta/indications_dosage/)

(http://www.rxlist.com/nucynta-drug.htm)

(Certificate of Analysis from MSN Organics Pvt. Ltd)

2.1.1 Tapentadol Hydrochloride

Molecular structure



Chemical name

3-[(1R, 2R)-3-(dimethylamino)-1-ethyl-2-methylpropyl]phenol monohydrochloride

Molecular formula $C_{14}H_{23}NO.HCl$

Molecular weight 257.8

Category Centrally acting opioid analgesic.

Description White to off white crystalline powder

Solubility Tapentadol Hdrochloride is highly soluble in methanol, water and insoluble in n-heptane.

Identification test

i) Melting point

Standard value	Observed value*
194-196˚C	194.33˚C

*****Mean of six observations

ii) IR spectrum was recorded and shown in figure 1.

Storage Preserve in well-closed containers at controlled room temperature.

pKa value 9.34 and 10.45

Mechanism of action

Tapentadol Hydrochloride have dual mode of action as an agonist at the μ-opioid receptor and as a nor-epinephrine reuptake inhibitor, there is potential for off label use in chronic pain.

Side effects

Nausea, dizziness, constipation, and CNS sedation are common side effects of opioid pain medications. Tapentadol Hydrochloride may cause hallucinations and short term memory loss to patients on anti-depressants.

Pharmacological action

Pharmacodynamics

Tapentadol is a centrally acting oral analgesic with a dual mechanism of action, combining μ-opioid receptor agonist and norepinephrine reuptake inhibition in a single molecule. Norepinephrine plays a role in the endogenous descending pain

inhibitory system, and the analgesic efficacy of norepinephrine reuptake inhibitors has been shown in neuropathic pain.

Analgesic effect of Tapentadol has been demonstrated in a wide range of animal models of pain with nociceptive and neuropathic components, and development of tolerance to its analgesic effect was twice as slow as that of morphine. Although Tapentadol has a 50-fold lower binding affinity to μ - opioid receptor, its analgesic potency is only 2 to 3 times lower than that of morphine, indicating that the dual mode of action may result in an opiate-sparing effect (Tzschentke et al 2007).

Pharmacokinetics

A study investigated the absorption, metabolism, and excretion of tapentadol in humans using oral 3-[14C]-labeled Tapentadol Hydrochloride (Terlinden et al 2007). Important findings were

- Drug absorption was rapid, reaching a maximum concentration of 2.45 microg-eq/mL in 1.25 to 1.5 hours.
- The drug was present mainly in the form of conjugated metabolites.
- Excretion was renal and complete within 5 days.

The absolute oral bioavailability of Tapentadol is approximately 32%, which is comparable to that of morphine (Tzschentke et al 2006).

2.2 REPORTED METHODS

2.2.1 Analytical Methods

2.2.1.1 James A. Bourland et al., (2010), reported **"Determination of Tapentadol and *N*-Desmethyltapentadol in Authentic Urine Specimens by Ultra-Performance Liquid Chromatography-Tandem Mass Spectrometry"** An Acquity UPLC was coupled with a tandem quadrupole detector (TQD) operating in positive ESI mode for analysis (Waters, Milford, MA). The UPLC column was a Waters

Acquity UPLC® BEH Shield RP18 (2.1 mm × 50 mm × 1.7 μm). The column manager temperature was held at 50°C, and the injection volume was 5 μL. The mobile phase was made up of 0.1% formic acid (Solvent A) and acetonitrile (Solvent B). The mobile phase composition initially was 90% A/10% B with a flow rate of 0.4 mL/min and during the cycle increased up to 90% acetonitrile by 1.50 min, returning to the original composition at 2.0 min, with a total cycle time between injections of 3.0 min. The source temperature was set to 150°C and desolvation temperature at 450°C. The desolvation gas was nitrogen and the collision gas was argon with flow rates of 500 L/h and 0.18 mL/min, respectively.

2.2.1.2 Cynthia Coulter et al**.,** (2010), reported **"Determination of Tapentadol and its Metabolite *N*-Desmethyltapentadol in Urine and Oral Fluid Using Liquid Chromatography with Tandem Mass Spectral Detection"** A 1200 series LC pump coupled to a 6410 triple-quadrupole MS, operating in positive electrospray ionization mode (ESI) mode was used for analysis (Agilent Technologies, Santa Clara, CA). The LC column (Agilent Technologies) was a Zorbax Eclipse XDB C18 (4.6 × 50 mm × 1.8 mm). The column temperature was held at 40°C. The injection volume for oral fluid was 5 μl; the injection volume for urine was 2 μl. The mobile phase consisted of 20 mm ammonium formate (pH 6.4) (Solvent A) and methanol (Solvent B). Initially, the mobile phase composition was 85% A:15% B at a flow rate of 0.7 ml/min. Over 4 min, the percentage of methanol was increased to 100%. The gas temperature was 350°C, the gas flow was 10 L/min, and the nebulizer pressure was 50 psi. Nitrogen was used as the collision gas, and the capillary voltage was 4000 V.

2.2.1.3 Ravikumar K et al., (2011), reported **"Four Stereoisomers of the Novel μ-Opioid Receptor Agonist Tapentadol Hydrochloride"** The crystal and molecular

structures of four stereoisomers of Tapentadol Hydrochloride [systematic name: 3-(3-hydroxyphenyl)-*N*,*N*,2-trimethylpentan-1-aminium chloride], $C_{14}H_{24}NO^{+}\cdot Cl^{-}$, a novel analgesic agent, have been determined by X-ray crystal structure analysis. Resolution of the isomers was carried out by reverse-phase and chiral high-performance liquid chromatographic (HPLC) methods. Stereoisomers (I) and (II) crystallize in the monoclinic space group $P2_1$, each with two Tapentadol cations and two chloride anions in the asymmetric unit, while stereoisomers (III) and (IV) crystallize in the orthorhombic space group $P2_12_12_1$, with one tapentadol cation and one chloride anion in the asymmetric unit. The absolute configurations of the four enantiomers were determined unambiguously by X-ray crystallography. The crystal structures reveal the stereochemistries at the 3-ethyl and 2-methyl groups to be *R*,*R*, *S*,*S*, *S*,*R* and *R*,*S* in stereoisomers (I)-(IV), respectively. The ethyl and aminopropyl groups adopt different orientations with respect to the phenol ring for (I) and (IV). In all four structures, the chloride ions take part in N-H----Cl and O-H----Cl hydrogen bonds with the tapentadol molecules, resulting in one-dimensional helical chains in the crystal packing in each case.

2.2.2 Clinical Methods

2.2.2.1 Schroder W et al., reported (2011**), "Synergistic Interaction Between the Two Mechanisms of Action of Tapentadol in Analgesia"** This study was designed to investigate the nature of the interaction of the two mechanisms (μ-opioid receptor (MOR) agonism and noradrenaline reuptake inhibition (NRI)). Pharmacological antagonism studies have demonstrated that both mechanisms of action contribute to the analgesic effects of Tapentadol. Dose-response curves were generated in rats for Tapentadol alone or in combination with the opioid antagonist naloxone or the α(2)-adrenoceptor antagonist yohimbine. Two different pain models were used:

1) low-intensity tail-flick and 2) spinal nerve ligation. Tapentadol produced dose-dependent analgesic effects in both pain models, and its dose-effect curves were shifted to the right by both antagonists, thereby providing data to distinguish between MOR agonism and NRI This may explain why Tapentadol is only 2- to 3-fold less potent than morphine across a variety of preclinical pain models despite its 50-fold lower affinity for the MOR. This is probably the first demonstration of a synergistic interaction between the occupied receptors for a single compound with two mechanisms of action.

2.2.2.2 Smit JW et al., (2010), reported **"Effect of Acetaminophen, Naproxen and Acetylsalicylic acid on Tapentadol Pharmacokinetics: Results of Two Randomized, Open-Label, Crossover, Drug-Drug Interaction Studies"**. In both randomized and crossover studies, Tapentadol immediate release (IR) 80 mg were administered as a single oral dose alone. In the 2-way crossover study, Tapentadol IR was also given with the fifth of seven doses of acetaminophen 1000 mg; in the 3-way crossover study, Tapentadol IR was also given with the third of four doses of naproxen 500 mg and the second of two doses of acetylsalicylic acid 325 mg. All treatments were separated by a washout period of 7-14 days. No clinically relevant changes were noted in the serum concentrations of Tapentadol, and accordingly, no dosage adjustments with respect to the investigated pharmacokinetic mechanism of interaction are warranted for the administration of Tapentadol given concomitantly with acetaminophen, naproxen, or acetylsalicylic acid.

2.2.2.3 Rolf Terlinden et al., (2007), reported **"Absorption, Metabolism, and Excretion of ^{14}C-Labeled Tapentadol Hydrochloride in Healthy Male Subjects".** Four healthy male subjects received a single 100-mg oral dose of 3-[^{14}C]-labeled Tapentadol Hydrochloride for evaluation of the pharmacokinetics of the drug and the

excretion balance of radiocarbon. The concentration-time profiles of radiocarbon in whole blood and serum and radiocarbon excretion in the urine and feces, and the expired carbon di oxide were determined. Absorption was rapid (with a mean maximum serum concentration [C_{max}], 2.45 μg-eq/m]; a time to C_{max}, 1.25–1.5 h), and the drug was present primarily in the form of conjugated metabolites (conjugated:unconjugated metabolites = 24:1). Excretion of radiocarbon was rapid and complete (>95% within 24 h; 99.9% within 5 days) and almost exclusively renal (99%:69% conjugates; 27% other metabolites; 3% in unchanged form). It was found that a single oral dose of Tapentadol was rapidly absorbed, then excreted into the urine, primarily in the form of conjugated metabolites, and was well tolerated.

2.2.2.4 Xu et al., (2010), reported **"Population Pharmacokinetics of Tapentadol Immediate Release (IR) in Healthy Subjects and Patients with Moderate or Severe Pain"** The analysis included pooled data from 11 385 serum pharmacokinetic samples from 1827 healthy subjects and patients with moderate to severe pain. The population pharmacokinetic model for Tapentadol IR identified the relationship between pharmacokinetic parameters and a wide range of covariates. The simulations of Tapentadol exposure with identified, statistically significant covariates demonstrated that only hepatic function (as characterized by total bilirubin and total protein) may be considered a clinically relevant factor that warrants dose adjustment. None of the other covariates are of clinical relevance, nor do they necessitate dose adjustment.

2.2.2.5 William E et al., (2010), reported **"Tapentadol Hydrochloride: A Centrally Acting Oral Analgesic"** The purpose of this article is to review animal studies, pharmacokinetic studies, drug-drug interaction studies, and Phase II/III trials of

Tapentadol in various conditions producing moderate to severe pain. Tapentadol appears to be a well-tolerated and effective analgesic for the treatment of moderate to severe acute pain. Although not currently approved for the management of chronic pain, Tapentadol has been reported to be effective in managing pain associated with osteoarthritis and low back pain.

2.2.2.6 Kwong WJ et al., (2010), reported **"Cost-Effective Analysis of Tapentadol Immediate Release for the Treatment of Acute Pain"** this analysis compared the cost – effectiveness of Tapentadol IR with doses of Oxycodone IR providing comparable analgesia in the outpatient treatment of acute postsurgical and nonsurgical pain. A markov model was developed to simulate clinical-economical outcomes for Tapentadol IR 100 mg compared with Oxycodone IR 15 mg in the treatment of acute postsurgical pain (3 days) and for Tapentadol IR 50 mg compared with Oxycodone IR 10 mg in the treatment of acute nonsurgical pain (10 days). The results of this model suggest that at doses providing comparable analgesia, Tapentadol IR is cost-effective alternative to Oxycodone IR for the treatment of acute surgical and nonsurgicl pain.

2.2.2.7 Raritan NJ et al(2008), reported **"FDA Approves Tapentadol Immediate Release Tablets for Relief of Moderate to Severe Acute Pain"**. Johnson & Johnson Pharrmaceutical research & development, L.L.C., announced that the U.S. food and drug administration approved Tapentadol immediate-release tablets for the relief of moderate to severe acute pain in adults 18 years of age or older. Tapentadol is a new centrally acting oral analgesic. It has two mechanisms of action, combining μ-opioid receptor agonism and norepinephrine reuptake inhibition. Tapentadol tablets have been approved in 50 mg, 75 mg, and 100 mg doses.

Aim &
Plan of Work

3. AIM AND PLAN OF WORK

3.1 AIM OF WORK

The drug analysis plays an important role in the development, manufacture and therapeutic use of drug. Most of the pharmaceutical industries do the quantitative chemical analysis to ensure that the raw material used and the final product thus obtained meet certain specifications and to determine how much of each components are present in the final product.

Standard analytical procedure for newer drugs or formulation may not be available in pharmacopoeias hence it is essential to develop newer analytical methods which are precise, accurate, specific, linear, simple and rapid. UV and HPLC grades of solvents used for respective determination and solvent should readily available and cheaper. The solvent should be completely extracting the active ingredient from formulation.

Tapentadol Hydrochloride is an opioid analgesic. Its main use is to treat the moderate to severe acute pain, and first approved in the USA for this purpose. This is a newer drug available in Indian market and CDSCO approves this on April 2011.

Extensive literature survey revealed that the determination of Tapentadol and N-desmethyltapentadol in authentic urine specimens by UPLC - Tandem Mass Spectrometry has been reported. However there is no evidence for the estimation of Tapentadol Hydrochloride by UV - Visible spectrophotometry and HPTLC in bulk and in tablet formulation.

Hence the present work, aims to develop a simple, precise and accurate methods for the estimation of Tapentadol Hydrochloride in bulk and in pharmaceutical dosage form by using UV spectroscopy, visible spectrophotometry and HPTLC and to validate the developed methods.

3.2 PLAN OF WORK

3.2.1 Survey on Literature

The survey on literature performed for Tapentadol Hydrochloride for their physiochemical properties, solubility, pharmacology and analytical techniques. So this basic information gives notation for newer method development.

3.2.2 Method Development

1) Identification of drug by its melting point and IR spectral studies.

2) Selection of suitable solvent for quantitative extraction of drug present in the formulations. The solvent should be readily available, economical and of analytical grade for UV-spectroscopy, visible spectrophotometry and HPTLC. The solvent should not interact with the compound of interest and its structural characteristics.

3) Selection of method for analysis, depending on the spectral characteristics of the drug.

4) Selection of suitable wavelength for rapid and accurate simple UV and Difference spectrophotometric methods development.

5) Development of simple, cost effective and accurate visible spectrophotometric methods.

6) Development of simple and accurate HPTLC method.

7) Analysis of marketed formulations.

8) Validation of developed analytical methods.

9) Statistical analysis of developed analytical methods.

3.2.3 Validation of Developed Method

The developed method should be validated as per ICH guidelines. The parameters used to validate the developed method are Specificity, Linearity, Range, Accuracy, Precision, Limit of Detection, Limit of Quantification, Robustness and Ruggedness.

Materials & Methods

4. MATERIALS AND METHODS

4.1 MATERIALS

4.1.1 Drug Samples (Raw material)

Tapentadol Hydrochloride was obtained as a gift sample from MSN Laboratories Limited, Hyderabad.

4.1.2 Formulation Used

Tapol-100 tablets containing Tapentadol Hydrochloride 100 mg was procured from MSN Laboratories Limited, Hyderabad.

4.1.3 Chemicals and Solvents Used

Distilled water, Sodium hydroxide (AR Grade), MBTH reagent (AR Grade), Hydrochloric acid (AR Grade), Ceric Ammonium Sulphate (AR Grade), Sulphuric acid (AR Grade), Potassium Ferricyanide (AR Grade), Ferric Chloride(AR Grade), Methanol (AR Grade), Ethyl Acetate (AR Grade), Hexane (AR Grade) were purchased from Qualigens India Pvt. Limited, Mumbai and Loba Chemie India Limited, Mumbai.

4.1.4 Instruments Used

Different instruments used to carry out the present work are

1) Shimadzu AUX - 220 Digital balance
2) Shimadzu - 1700 Double Beam UV - Visible spectrophotometer with pair of 10 mm matched quartz cells
3) ELICO SL - 210 Double Beam UV - Visible spectrophotometer with pair of 10 mm matched quartz cells
4) HPTLC Instrument (Camag)
5) ELICO pH meter (Model LI - 120)
6) SOLTEC – Sonica ultrasonic cleaner – Model 2200 MH
7) REMI – Centrifuge apparatus

8) CYBERLAB – Micropipette

9) SUNBIM – Melting point apparatus

4.1.5 Specifications (Terms) of instruments

4.1.5.1 Shimadzu AUX - 220 Digital Balance (Shimadzu Instruction Manual)

Specifications	
Weighing capacity	200 gms
Minimum display	0.1 mg
Standard deviation	≤ 0.1 mg
Operation temperature range	5 to 40° C

4.1.5.2 Double Beam UV - Visible Spectrophotometer (Shimadzu and ELICO Instruction Manuals)

Model: Shimadzu UV - 1700; Double beam UV - Visible spectrophotometer.

ELICO SL – 210; Double beam UV - Visible spectrophotometer.

Specification	**Shimadzu UV - 1700**	**Elico SL – 210**
Light source	20 W halogen lamp, Deuterium lamp, Light source position automatic adjustment mechanism. Built in lamp lighting time display function.	Tungsten halogen lamp (W), Deuterium lamp (D), Light source position automatic adjustment mechanism.

Monochromator	Aberration- correcting concave blazed holographic grating	Concave holographic grating with 1200 lines/ mm
Detector	Silicon photodiode	Photodiode
Stray Light	0.04% or less (220 nm; NAI 10g/ lt) 0.04% or less (340 nm; $NaNo_2$ 50g/ lt).	< 0.05% T at 220 nm with NAI 10g/ lt
Measurement Wavelength range	190 ~ 1100 nm	190 ~1100 nm
Spectral Band width	1 nm or less (190 to 900nm).	1.8 nm
Wave length Accuracy	± 0.5 nm on broad automatic wavelength calibration mechanism.	± 0.5 nm automatic wavelength calibration mechanism.
Recording range	Absorbance; - 3.99 ~ 3.99 Abs Transmittance; - 399 ~ 399%	Absorbance; ± 3.000 Abs
Photometric accuracy	± 0.004 Abs (at 1.0 Abs). ± 0.002 Abs (at 0.5 Abs).	± 0.005 Abs (at 1.0 Abs). ± 0.010 Abs (at 0.5 Abs).
Operating Temperature/	Temperature range; 15 to 35˚C	Temperature range; 15 to 35˚C

Humidity	Humidity range; 35 to 80% (15 to below 35˚C) 35 to 70% (30 to below 35˚C)	Humidity range; 35 to 80% (15 to below 35˚C) 35 to 70% (30 to below 35˚C)

4.1.5.3 HPTLC instrument – Camag

Specifications	
Sample Applicator	Linomat IV with Camag100µl Syringe
Chamber	Camag Twin trough Glass Chamber (20x10)
Scanner	Camag TLC Scanner III
Software	Wincat software
Development temp	Ambient
Stationary Phase	HPTLC plates(Merck) precoated with silicagel 60 F254 on aluminium sheets
Development time	20 min

4.2 METHODS

In the present work an attempt was made to develop and validate simple, precise and accurate methods for the estimation of Tapentadol Hydrochloride in pure form and in tablet dosage form. The methods developed were the following

1) UV spectroscopy
2) Difference spectrophotomety
3) Visible spectrophotometry
    a) Method – I (using MBTH reagent)
    b) Method – II (using potassium ferricyanide)
4) HPTLC

4.2.1 UV Spectroscopic Method

4.2.1.1 Selection of solvent

The solubility of Tapentadol Hydrochloride was determined in a variety of solvents as per Indian pharmacopoeia standards. Solubility was carried out in polar and non polar solvents. From the solubility data distilled water was selected as solvent for the analysis of Tapentadol Hydrochloride.

4.2.1.2 Preparation of standard stock solution

25 mg of Tapentadol Hydrochloride raw material was weighed accurately and transferred in to 25 ml volumetric flask, dissolved in distilled water and made up to the volume with distilled water. This solution contains 1mg/ ml concentration.

4.2.1.3 Selection of wavelengths for estimation and stability studies

The standard stock solution was further diluted with distilled water to get the concentration of 10 µg/ ml and the solution was scanned between 200 and 400 nm using distilled water as blank. From the spectra, λmax was found to be 272.5 nm and was selected as an analytical wavelength.

The stability was performed by measuring the solution at different time intervals. It was observed that Tapentadol Hydrochloride in distilled water was stable up to 24 hours at the selected wavelength.

4.2.1.4 Preparation of calibration graph

The standard stock solution of Tapentadol Hydrochloride (1–6 ml) was transferred into a series of 100 ml volumetric flasks and made up to the volume with distilled water. The absorbance of different concentration solutions were measured at 272.5 nm. The calibration curve was constructed by plotting concentration Vs absorbance. Tapentadol Hydrochloride was linear with the concentration range of 10 - 60 μg/ ml at 272.5 nm.

4.2.1.5 Quantification of raw material

2.0 ml of standard stock solution was taken into a series of six 100 ml standard flasks and the volume was made up to mark with distilled water. The absorbance of these solutions was measured at 272.5 nm. The amount Tapentadol Hydrochloride present in the raw material was determined by using slope and intercept values from calibration graph.

4.2.1.6 Quantification of formulation

Ten tablets of formulation (Tapol-100 containing Tapentadol Hydrochloride equivalent to 100 mg) were weighed accurately and the average weight of each tablet was found. The tablets were ground to a fine powder. The tablet powder equivalent to 25 mg of Tapentadol Hydrochloride was weighed and transferred into 25 ml volumetric flask. Added about 20 ml of distilled water to dissolve the substance and the solution was sonicated for 15 minutes. Then it was made up to the volume to 25 ml with distilled water (1 mg/ ml) and centrifuged for 15 minutes. The supernatant liquid was filtered through Whatmann filter paper No. 41. From the clear solution, further dilutions were

made by diluting 2.0 ml into 100 ml with distilled water to obtain 20 µg/ ml solution theoretically. The absorbances of six replicates were measured and the amount was calculated by using regression equation. This procedure was repeated for six times.

4.2.1.7 Recovery studies

4.2.1.7.1 *Preparation of Tapentadol Hydrochloride raw material stock solution*

250 mg of Tapentadol Hydrochloride was accurately weighed and transferred into 25 ml Volumetric flask and sufficient distilled water was added to dissolve the substance and made up to the mark with the same. This contains 10 mg/ ml concentration.

4.2.1.7.2 *Procedure*

The recovery experiment was done by adding known concentrations of raw material stock solution of Tapentadol Hydrochloride to the pre-analyzed formulation. The tablet powder equivalent to 25 mg of Tapentadol Hydrochloride was weighed accurately and added 2 ml, 2.5 ml, 3 ml of above raw material stock solution into a series of 25 ml volumetric flasks and dissolved with distilled water and sonicated for 15 minutes. The solution was made up to 25 ml with distilled water and centrifuged for 15 minutes at 2000 rpm. The supernatant liquid was filtered through a Whatmann filter paper No.41. 2 ml of the clear solution was transferred into 100 ml volumetric flask and made up to 100 ml with distilled water. The absorbance of three replicates was measured at the selected wavelength. The amount of drug recovered from formulation was calculated. The procedure was repeated for three times for each concentration.

4.2.1.8 Validation of developed method

4.2.1.8.1 *Linearity*

A calibration curve was plotted between concentration and absorbance. Tapentadol Hydrochloride was linear in the concentration range of 10 - 60 µg/ ml at 272.5 nm. The optical characteristics were calculated.

4.2.1.8.2 *Precision*

The repeatability of the method was confirmed by the analysis of formulation was repeated for six times with the same concentration. The amount of drug present in the tablet formulation was calculated. The percentage RSD and confidence interval were calculated.

The intermediate precision of the method was confirmed by intraday and inter day analysis i.e. the analysis of formulation was repeated three times in the same day and on three successive days. The amount of drugs present in the formulation was determined. The percentage RSD and confidence interval were calculated.

4.2.1.8.3 *Accuracy*

Accuracy of the method was confirmed by recovery studies. To the pre-analyzed formulation, a known quantity of raw material of Tapentadol Hydrochloride was added and the procedure was followed as per the analysis of formulation. The amount of each drug recovered was calculated. This procedure was repeated for three times for each concentration. The % RSD and confidence interval were calculated.

4.2.1.8.4 *Ruggedness*

Ruggedness of the method was confirmed by the analysis of formulation was done by using different instruments and different analysts. The amount was calculated. The % RSD and confidence interval were calculated.

4.2.1.8.5 *LOD and LOQ*

The limit of detection (LOD) is the lowest concentration at which the results still satisfy some predetermined acceptance criteria. Below the LOD, the results fail to meet these criteria (analysis is not feasible). It may expressed as

$$LOD = \frac{3.3\ \sigma}{S}$$

Where, σ = the standard deviation of the response

S = the slope of the calibration curve

The limit of quantitation (LOQ) is set at a higher concentration than LOD, in the statistical method, it is 10 SD above the mean blank value, this presenting a greater probability that a value at the LOQ is real and not just a random fluctuation of the blank reading.

$$LOQ = \frac{10\ \sigma}{S}$$

Where, σ = the standard deviation of the response

S = the slope of the calibration curve

4.2.2 Difference Spectrophotomeric Method

The difference spectrophotometric method is based on the measurement of amplitude between maxima and minima in two eqimolar solutions of the analyte in different chemical forms, which exhibit different spectral characteristics. The method is advantageous over others, as it achieves the spectrophotometric isolation of the drug; moreover, interference due to additives can be nullified as can be proved by no change in isobestic points.

4.2.2.1 Selection of solvent

Distilled water, 0.1M hydrochloric acid and 0.1M sodium hydroxide were selected as the solvent after considering the solubility and stability factors of the drug as well as excipients present in tablet formulation.

4.2.2.2 Preparation of standard stock solution

30 mg of Tapentadol Hydrochloride was accurately weighed and transferred in to 100 ml volumetric flask and added about 80 ml of distilled water to dissolve the drug. After the immediate dissolution, the volume was made up to the mark with distilled water. The solution was observed to contain 300 µg / ml.

4.2.2.3 Selection of wave length for estimation and stability studies

The selection of wavelengths for the estimation of Tapentadol Hydrochloride was done by preparing a solution containing 10 µg/ ml of Tapentadol Hydrochloride in 0.1M sodium hydroxide and 0.1M hydrochloric acid separately. The difference spectra were recorded by taking Tapentadol Hydrochloride in 0.1M hydrochloric acid as blank and Tapentadol Hydrochloride in 0.1M sodium hydroxide as sample. From the difference spectra, 290 nm and 269.5 nm were selected as maxima and minima, respectively. The measured value is the amplitude between the two equimolar solutions of the analyte in difference chemical forms, which exhibits different spectral characteristics. Tapentadol hydrochloride in 0.1M sodium hydroxide was stable up to 4 hours and in 0.1M hydrochloric acid was stable up to $3^{1}/_{2}$ hours.

4.2.2.4 Preparation of calibration graph

The aliquots of the 300 µg/ ml standard stock solution of Tapentadol Hydrochloride in distilled water (1 to 6 ml) were transferred in to two sets of series of 100 ml volumetric flasks. The first set of Tapentadol Hydrochloride solutions were diluted to 100 ml with 0.1N Hydrochloric acid, and the second set of Tapentadol

Hydrochloride solutions were diluted to 100 ml with 0.1N Sodium hydroxide. Difference spectrum was recorded by placing same concentration of acidic and basic solutions in reference and sample cells respectively. The procedure was repeated for six times, the amplitude was plotted against concentration.

4.2.2.5 Quantification of raw material

Six times the concentration of Tapentadol Hydrochloride raw material solution (12 µg/ ml) was prepared from the stock solution in 0.1M sodium hydroxide and 0.1M hydrochloric acid. Tapentadol Hydrochloride (12 µg/ ml) in 0.1M sodium hydroxide is kept in sample cell and Tapentadol Hydrochloride (12 µg/ ml) in 0.1M hydrochloric acid is kept in reference cell. The amplitude between the equimolar concentrations was measured. The concentration of Tapentadol Hydrochloride was determined by using slope and intercept values from calibration values.

4.2.2.6 Quantification of formulation

Twenty tablets (Tapol-100 containing Tapentadol Hydrochloride equivalent to 100 mg) were weighed accurately and the average weight was found. The tablets were ground to a fine powder. The weight of tablet pwder equivalent to 30 mg of Tapentadol Hydrochloride was taken in 100 ml volumetric flask. It was then dissolved in distilled water by intermittent shaking for 4 – 5 minutes. The solution was sonicated for 15 minutes. Then it was made up to the volume 100 ml with distilled water, and centrifuged at 2000 rpm for 15 minutes and the solution were filtered through whatman filter paper (No.41).

4.2.2.6.1 Assay procedure

Six times the concentration of Tapentadol Hydrochloride test solution (12 µg/ ml) was prepared from the above solution (4 ml into 100 ml) in 1M sodium hydroxide and in 0.1M hydrochloric acid. Tapentadol Hydrochloride (12 µg/ ml) in

0.1M hydrochloric acid was kept in reference cell and Tapentadol Hydrochloride (12 µg/ ml) in 0.1M sodium hydroxide was kept in sample cell. The amplitude between the equimolar solutions was measured. The concentration of Tapentadol Hydrochloride was determined by using slope and intercept values from calibration graph.

4.2.2.7 Recovery studies

4.2.2.7.1 *Preparation of Tapentadol Hydrochloride raw material stock solution*

150 mg of Tapentadol Hydrochloride raw material was accurately weighed and 10 ml Volumetric flask and sufficient distilled water was added to dissolve the substance and made up to the mark with the same. This contains 15 mg/ ml concentration.

4.2.2.7.2 *Procedure*

The recovery experiment was done by adding known concentrations of raw material stock solution of Tapentadol Hydrochloride to the preanalyzed formulation. The tablet powder equivalent to 30 mg of Tapentadol Hydrochloride was weighed accurately and added 1.5 ml, 2.0 ml and 2.5 ml of above raw material stock solution into a series of 100 ml volumetric flasks and dissolved with distilled water, sonicated for 15 minutes. The solution was made up to 100 ml with distilled water and centrifuged for 15 minutes at 2000 rpm. The supernatant liquid was filtered through a Whatmann filter paper No.41. From the above solution 2.0 ml was transferred into two 100 ml volumetric flasks and one was made up to 100 ml with 0.1N sodium hydroxide and another one was made up with 0.1N hydrochloric acid. The procedure was reported as per the analysis of formulation. This procedure was repeated for three times for each concentration.

4.2.2.8 Validation of developed method

4.2.2.8.1 *Linearity*

A calibration curve was plotted between concentration and amplitude. Tapentadol Hydrochloride was linear with the concentration range of 3 - 18 µg/ ml.

4.2.2.8.2 *Precision*

The repeatability of the method was confirmed by the analysis of formulation was repeated for six times with the same concentration. The amount of drug present in the tablet formulation was calculated. The percentage RSD and confidence interval were calculated.

The intermediate precision of the method was confirmed by intraday and inter day analysis i.e. the analysis of formulation was repeated three times in the same day and on three successive days. The amount of drugs was determined. The percentage RSD and confidence interval were calculated.

4.2.2.8.3 *Accuracy*

Accuracy of the method was confirmed by recovery studies. To the pre-analyzed formulation, a known quantity of raw material of Tapentadol Hydrochloride was added and the procedure was followed as per the analysis of formulation. The amount of each drug recovered was calculated. This procedure was repeated for three times for each concentration. The % RSD and confidence interval were calculated.

4.2.2.8.4 *Ruggedness*

Ruggedness of the method was confirmed by the analysis of formulation was done by using different instruments and different analysts. The amount was calculated. The % RSD and confidence interval were calculated.

4.2.2.8.5 ***LOD and LOQ***

The limit of detection (LOD) is the lowest concentration at which the results still satisfy some predetermined acceptance criteria. Below the LOD, the results fail to meet these criteria (analysis is not feasible). It may expressed as

$$LOD = \frac{3.3\ \sigma}{S}$$

Where, σ = the standard deviation of the response

S = the slope of the calibration curve

The limit of quantitation (LOQ) is set at a higher concentration than LOD, in the statistical method, it is 10 SD above the mean blank value, this presenting a greater probability that a value at the LOQ is real and not just a random fluctuation of the blank reading.

$$LOQ = \frac{10\ \sigma}{S}$$

Where, σ = the standard deviation of the response

S = the slope of the calibration curve

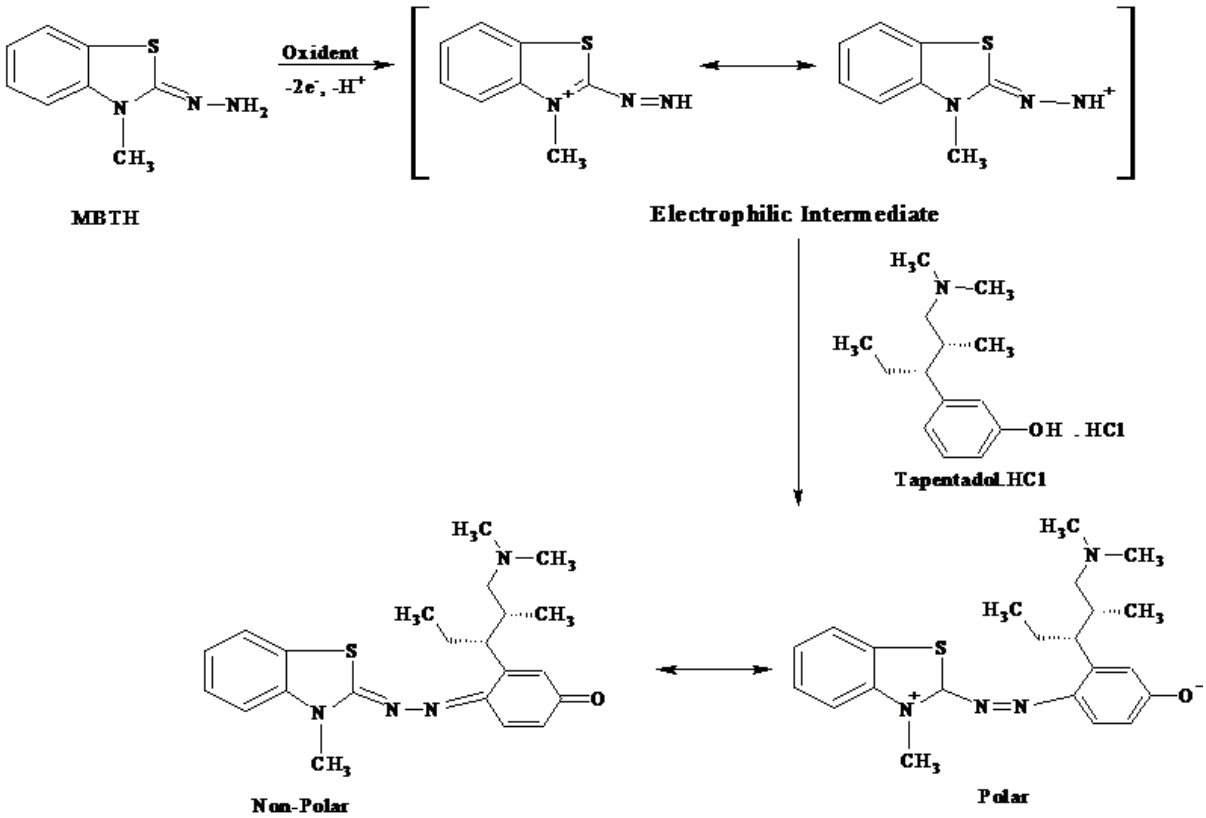
4.2.3 Visible Spectrophotometric Methods

4.2.3.1 Colorimetric method - I

4.2.3.1.1 Principle (Burudi kalyanaramu et al., 2011; Yuan Cun-guang, 1988)

MBTH is a 3-Methyl-2-benzothiazolinone hydrazone hydrochloride monohydrate. MBTH reagnt is used for the estimation of drugs contain phenols, aromatic amines, aldehydes, polyhydroxy compounds, indoles, phenothiazones. With Phenols under reaction condition MBTH loses two electrons and one proton to form the electrophilic intermediate, which has been identified as the active coupling species that

undergoes electrophilic substitution with phenol and other groups to form the colored product. Para un-substituted phenols form orange red colour and para substituted phenols forms violet green with less intensity. Violet green coloured products are less



stable than orange red coloured product.

Figure: Schematic Reaction of Tapentadol Hydrochloride With MBTH Reagent

Tapentadol Hydrochloride have the para un-substituted phenol, and it react with MBTH reagent in the presence of oxidizing agent ceric ammonium sulphate in 1M sulphuric acid and forms an orange red colored complex is formed which can be estimated by colorimetrically at 514 nm.

4.2.3.1.2 Preparation of reagents

0.7% w/v of the MBTH was prepared by weighing 700 mg of the MBTH powder in to 100 ml volumetric flask, dissolved in 60 ml of water to dissolve and then made up to 100 ml with distilled water.

0.01M ceric ammonium sulphate solution is prepared by weighing 0.6326 gm of ceric ammonium sulphate in to 100 ml volumetric flask and dissolved in 1M sulphuric acid and made up to the mark with 1M sulphuric acid.

4.2.3.1.3 Preparation of standard stock solution

10 mg of the Tapentadol Hydrochloride raw material was transferred in to 100 ml volumetric flask, dissolved in distilled water and made up to the volume to 100 ml with distilled water. The solution contains 100 µg/ ml of Tapentadol Hydrochloride.

4.2.3.1.4 Selection of wavelength for estimation and stability studies

2.5ml of the standard stock solution of Tapentadol Hydrochloride pipetted out in to 25 ml standard flask. To this added 1ml of 0.01M ceric ammonium sulphate solution and shaken well then added 0.8 ml of 0.7%w/v MBTH reagent with shaking. The volume was made up to the mark with distilled water to get concentration of 10 µg/ ml. The orange red coloured chromogen was scanned in visible region (400 – 800 nm) against reagent blank. From the spectra the λmax selected was 514 nm.

The stability was performed by measuring the absorbance of same solution at different time intervals. It was observed that Tapentadol Hydrochloride was stable up to four hours fifteen minutes at the selected wavelength.

4.2.3.1.5 Optimization of reagents

The absorbance of Tapentadol Hydrochloride in different strengths and volumes of both ceric ammonium sulphate and MBTH reagent were optimized to get steady absorbance.

The molarity of ceric ammonium sulphate was optimized by taking 2.5 ml of standard drug solution in to separate 25 ml volumetric flasks and added 1 ml of different molar solution of (0.002M - 0.02M) ceric ammonium sulphate and constant volume of 0.8 ml of 0.7% w/v MBTH reagent were added with shaking and made up to

the volume with distilled water. The absorbance of the solution in each standard flask was measured at 514 nm against reagent blank.

The volume of ceric ammonium sulphate solution was optimized by taking 2.5 ml of standard drug solution (10 µg/ ml) in to separate 25 ml volumetric flasks and (0.1ml/ 1.5ml) of constant molarity of 0.01M ceric ammonium sulphate solution was added and constant volume of 0.8 ml of 0.7% w/v MBTH reagent were added with shaking and made up to the volume with distilled water. The absorbance of the solution in each standard flask was measured at 514 nm against reagent blank.

The strength of MBTH reagent was optimized by taking 2.5 ml of standard drug solution (10 µg/ ml) in to separate 25 ml volumetric flasks, added a constant volume of 1ml of 0.01M ceric ammonium sulphate and 0.8 ml of MBTH reagent of each strength (0.1% - 1.0%). The solutions were shaking and made up to the volume with distilled water. The absorbance of the solution in each standard flask was measured at 514 nm against reagent blank.

The volume of the MBTH reagent was optimized by taking 2.5 ml of standard drug solution in to separate 25 ml volumetric flasks in which constant volume of 1 ml of 0.01M ceric ammonium sulphate and (0.1 ml-1.2 ml) of 0.7% w/v of MBTH reagent were added with shaking and made up to the volume with distilled water. The absorbance of the solution in each standard flask was measured at 514 nm against reagent blank.

It was found that 1.0 ml of 0.01M ceric ammonium sulphate and 0.8 ml of 0.7% w/v MBTH reagent were showed marked absorbance.

4.2.3.1.6 Preparation of calibration graph

The standard stock solutions of Tapentadol Hydrochloride (0.5 ml – 6 ml) were transferred into a series of 25 ml volumetric flasks. To each flask, 1 ml of ceric

ammonium sulphate and 0.8 ml of 0.7% w/v of MBTH reagent were added with shaking and made up to the volume with distilled water. The absorbance of different concentration solutions were measured at 514 nm. The calibration curve was plotted. Tapentadol Hydrochloride was linear with the concentration range of 2-24 µg/ ml at 514 nm.

4.2.3.1.7 Quantification of raw material

2.5 ml of standard stock solution of Tapentadol Hydrochloride was pipetted into a series of six 25 ml volumetric flasks, added 1 ml of ceric ammonium sulphate, 0.8 ml of 0.7% w/v of MBTH reagent, shaken well and the volume was made up to the mark with distilled water. The absorbances of the solutions were measured at 514 nm. This procedure was repeated for six times.

4.2.3.1.8 Quantification of formulation

Twenty tablets of formulation (Tapol-100 containing Tapentadol Hydrochloride equivalent to 100 mg) were weighed accurately and the average weight of each tablet was found. The tablets were ground to a fine powder. The tablet powder equivalent to 10 mg of Tapentadol Hydrochloride was weighed and transferred into 100 ml volumetric flask, added about 80 ml of distilled water to dissolve the substance and sonicated for 15 minutes. The solution was made up to the volume with distilled water (100 µg/ ml). The solution was centrifuged for 15 minutes at 2000 rpm and filtered through Whatmann filter paper No. 41. From the clear solution 2.5 ml was pipetted out into a series of six 25 ml volumetric flasks, added 1 ml of ceric ammonium sulphate, 0.8 ml of 0.7% w/v of MBTH reagent with shaking and volume was made up to the mark with distilled water. The absorbances of the solutions were measured at 514 nm. This procedure was repeated for six times.

4.2.3.1.9 Recovery

4.2.3.1.9.1 *Preparation of Tapentadol Hydrochloride raw material stock solution*

An accurately weighed quantity of 40 mg of Tapentadol Hydrochloride was transferred into 10 ml volumetric flask, added sufficient distilled water to dissolve the substance and made up to the volume with distilled water. The solution contains 4 mg/ ml of Tapentadol Hydrochloride.

4.2.3.1.9.2 *Procedure*

The recovery experiment was done by adding known concentrations of raw material stock solution of Tapentadol Hydrochloride to the preanalyzed formulation. To the tablet powder equivalent to 10 mg of Tapentadol Hydrochloride, added 2 ml, 2.5 ml, and 3 ml of above raw material stock solution in to a series of three 100 ml standard flasks, dissolved in distilled water and sonicated for 15 minutes. The solution was made up to 100 ml with distilled water. The solution was centrifuged for 15 minutes at 2000 rpm and the solution was filtered through a Whatmann filter paper No.41. 2.5 ml of the clear solution was transferred in to a series of six 25 ml volumetric flasks, added 1 ml of ceric ammonium sulphate, 0.8 ml of 0.7% w/v of MBTH reagent and the volume was made up to the mark with distilled water. The absorbances of these solutions were measured at 514 nm. This procedure was repeated for three times.

4.2.3.1.10 Validation of developed method

4.2.3.1.10.1 *Linearity*

A calibration curve was plotted between concentration and amplitude. Tapentadol Hydrochloride was linear with the concentration range of 2 - 24 µg/ ml.

4.2.3.1.10.2 *Precision*

The repeatability of the method was confirmed by the analysis of formulation was repeated for six times with the same concentration. The amount of drug present in

the tablet formulation was calculated. The percentage RSD and confidence interval were calculated.

The intermediate precision of the method was confirmed by intraday and inter day analysis i.e. the analysis of formulation was repeated three times in the same day and on three successive days. The amount of drugs was determined. The percentage RSD and confidence interval were calculated.

4.2.3.1.10.3 ***Accuracy***

Accuracy of the method was confirmed by recovery studies. To the pre-analyzed formulation, a known quantity of raw material of Tapentadol Hydrochloride was added and the procedure was followed as per the analysis of formulation. The amount of each drug recovered was calculated. This procedure was repeated for three times for each concentration. The % RSD and confidence interval were calculated.

4.2.3.1.10.4 ***Ruggedness***

Ruggedness of the method was confirmed by the analysis of formulation was done by using different instruments and different analysts. The amount was calculated. The % RSD and confidence interval were calculated.

4.2.3.1.10.5 ***LOD and LOQ***

The limit of detection (LOD) is the lowest concentration at which the results still satisfy some predetermined acceptance criteria. Below the LOD, the results fail to meet these criteria (analysis is not feasible). It may expressed as

$$LOD = \frac{3.3\,\sigma}{S}$$

Where, σ = the standard deviation of the response

S = the slope of the calibration curve

The limit of quantitation (LOQ) is set at a higher concentration than LOD, in the statistical method, it is 10 SD above the mean blank value, this presenting a greater probability that a value at the LOQ is real and not just a random fluctuation of the blank reading.

$$LOQ = \frac{10\,\sigma}{S}$$

Where, σ = the standard deviation of the response

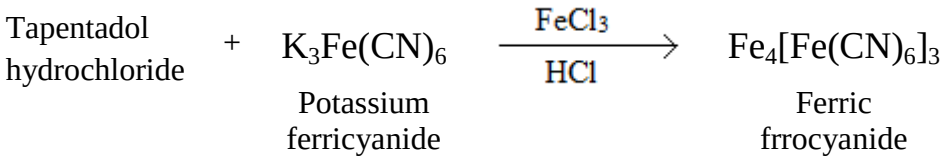
S = the slope of the calibration curve

4.2.3.2 Colorimetric method-II

4.2.3.2.1 Principle (Adhikamsetty R. K. et al., 2009)

Tapentadol Hydrochloride in acidic medium combines with potassium ferricyanide and ferric chloride forms water insoluble coloured complex called prussion blue with maximum absorbance at 737 nm. This Prussian blue colored substance is insoluble in acidic media but it is soluble in alkali.

4.2.3.2.1.1 Reaction



4.2.3.2.2 Preparation of reagent

0.5% w/v of the ferric chloride was prepared by weighing 500 mg of the ferric chloride in to 100 ml volumetric flask and dissolved in distilled water to dissolve and then made up to 100 ml with distilled water.

0.1% w/v potassium ferricyanide solution is prepared by weighing 100 gm of potassium ferricyanide in to 100 ml volumetric flask and dissolved in distilled water and made up to 100 ml with distilled water.

1M hydrochloric acid may be prepared by diluting 8.5 ml of hydrochloric acid to 100 ml with distilled water. (I.P 2007)

4.2.3.2.3 Preparation of standard stock solution

100 mg of the Tapentadol Hydrochloride raw material was transferred in to 100 ml volumetric flask, about 80 ml of distilled water was added to dissolve the substance and made up to 100 ml with distilled water. The solution contains 1 mg/ ml of Tapentadol Hydrochloride.

4.2.3.2.4 Preparation of working standard solution

From the stock solution, 5 ml was pipetted out in to a 100 ml volumetric flask. The volume was made up to 100 ml with distilled water. The solution contains 50 µg/ ml of Tapentadol Hydrochloride solution.

4.2.3.2.5 Selection of wavelength for estimation and stability studies

2 ml of the working standard solution of Tapentadol Hydrochloride was pipetted out in to 25 ml volumetric flask. To this 1.5 ml of 0.5% w/v solution of ferric chloride solution was added and shaken well for 2 minutes. Then 3.2 ml of 0.1% w/v solution of potassium ferricyanide was added, shaken well and kept aside for another 2 minutes. To this solution, 2.5 ml of 1M hydrochloric acid was added and allowed to stand for further 2 minutes. The volume was made up to the mark with distilled water to get concentration of 10 µg/ ml. The spectrum was recorded in the range of 400 – 800 nm against reagent blank. From the spectra the wavelength (λmax) selected was 737 nm.

The stability was performed by measuring the absorbance of the same solution at different time intervals. It was observed that Tapentadol Hydrochloride was stable up to five hours at the selected wavelength.

4.2.3.2.6 Optimization of reagents

The absorbance of Tapentadol Hydrochloride in different strengths and volumes of ferric chloride, potassium ferricyanide and 1M hydrochloric acid were measured to optimize the reagents.

The strength of ferric chloride was optimized by taking 2 ml of working standard solution in to separate 25 ml volumetric flasks and added 1.5 ml of different concentrations of (0.1%-1.0%) ferric chloride and shaken well for 2 minutes. Then 3.2 ml of 0.1%w/v solution of potassium ferricyanide was added and allowed to stand for 2 minutes. To this, 2.5 ml of 1M hydrochloric acid was added and kept aside for another 2 minutes, and made up to 25 ml with distilled water. The absorbance of the solution in each flask was measured at 737 nm against reagent blank.

The volume of 0.5% w/v ferric chloride solution was optimized by taking 2 ml of working standard drug solution (4 µg/ ml) in to separate 25 ml volumetric flasks and added 0.5% w/v solution of ferric chloride in different volumes (0.1 ml – 2 ml) and shaken for 2 minutes. Then 3.2 ml of 0.1% w/v solution of potassium ferricyanide was added, shaken well and kept aside for another 2 minutes. To this solution, 2.5 ml of 1M hydrochloric acid was added and allowed to stand for further 2 minutes, and made up to 25 ml with distilled water. The absorbance of the solution in each flask was measured at 737 nm against reagent blank.

The strength of potassium ferricyanide was optimized by taking 2 ml of working standard drug solution (4 µg/ml) in to separate 25 ml volumetric flasks, added a constant volume of 1.5 ml of 0.5% w/v solution of ferric chloride and shaken for 2 minutes. Then 3.2 ml of different strengths of (0.01% - 0.15%) potassium ferricyanide solution were added, shaken well and kept aside for another 2 minutes. To this solution, 2.5 ml of 1M hydrochloric acid was added and allowed to stand for further 2 minutes,

and made up to 25 ml with distilled water. The absorbance of the solution in each flask was measured at 737 nm against reagent blank.

The volume of the of 0.1% w/v solution of potassium ferricyanide was optimized by taking 2 ml of working standard drug solution (4 µg/ ml) in to separate 25 ml volumetric flasks and added a constant volume of 1.5 ml of 0.5% w/v solution of ferric chloride and shaken for 2 minutes. Added 0.1% w/v solution of potassium ferricyanide in different volumes (0.2 ml-4 ml), shaken well and kept aside for another 2 minutes. To this solution, 2.5 ml of 1M hydrochloric acid was added and allowed stand for further 2 minutes, and made up to 25 ml with distilled water. The absorbance of the solution in each flask was measured at 737 nm against reagent blank.

The strength of hydrochloric acid was optimized by taking 2 ml of working standard drug solution (4 µg/ml) in to separate 25 ml volumetric flasks and added a constant volume of 1.5 ml of 0.5% w/v solution of ferric chloride and shaken for 2 minutes. Then 3.2 ml of 0.1% w/v solution of potassium ferricyanide was added, shaken well and kept aside for another 2 minutes. To this solution, added 2.5 ml of each strength of (0.2M-2.0M) hydrochloric acid and allowed stand for further 2 minutes, and made up to 25 ml with distilled water. The absorbance of the solution in each flask was measured at 737 nm against reagent blank.

The strength of hydrochloric acid was optimized by taking 2 ml of working standard drug solution (4 µg/ml) in to separate 25 ml volumetric flasks and added a constant volume of 1.5 ml of 0.5% w/v solution of ferric chloride and shaken for 2 minutes. Then 3.2 ml of 0.1% w/v solution of potassium ferricyanide was added, shaken well and kept aside for another 2 minutes. To this solution, added different volumes of (0.5ml-5.0ml) 1M hydrochloric acid and allowed to stand for further

2 minutes, and made up to 25 ml with distilled water. The absorbance of the solution in each flask was measured at 737 nm against reagent blank.

It was found that 1.5 ml of 0.5% w/v solution of ferric chloride, 3.2 ml of 0.1% w/v solution of potassium ferricyanide and 2.5 ml of 1M hydrochloric acid were showed marked absorbance.

4.2.3.2.7 Preparation of calibration graph

The working standard solutions of Tapentadol Hydrochloride (0.5ml – 5ml) were transferred into a series of 25 ml volumetric flasks. To each flask, 1.5 ml of 0.5% w/v solution of ferric chloride and shaken for 2 minutes. Then 3.2 ml of 0.1% w/v solution of potassium ferricyanide was added, shaken well and kept aside for another 2 minutes. To this solution, 2.5 ml of 1M hydrochloric acid was added and allowed to stand for further 2 minutes, and made up to 25 ml with distilled water. The absorbance of different concentration solutions were measured at 737 nm. The calibration curve was plotted. Tapentadol Hydrochloride was linear with the concentration range of 1-10 µg/ ml at 737 nm.

4.2.3.2.8 Quantification of raw material

2 ml of working standard solution of Tapentadol Hydrochloride was pipetted into a series of six 25 ml volumetric flasks, added 1.5 ml of 0.5% w/v solution of ferric chloride and shaken for 2 minutes. Then 3.2 ml of 0.1% w/v solution of potassium ferricyanide was added, shaken well and kept aside for another 2 minutes. To this solution, 2.5 ml of 1M hydrochloric acid was added and allowed to stand for further 2 minutes, and made up to 25 ml with distilled water. The absorbances of the solutions were measured at 737 nm. This procedure was repeated for six times.

4.2.3.2.9 Quantification of formulation

Twenty tablets of formulation (Tapol-100 containing Tapentadol Hydrochloride equivalent to 100 mg) were weighed accurately and the average weight of each tablet was found. The tablets were ground to a fine powder. The tablet powder equivalent to 100 mg of Tapentadol Hydrochloride was weighed and transferred into 100 ml volumetric flask, added about 80 ml of distilled water to dissolve the substance and sonicated for 15 minutes. The solution was made up to the volume with distilled water (100 µg/ ml). The solution was centrifuged for 15 minutes at 2000 rpm and filtered through Whatmann filter paper No. 41. From the clear solution, 5 ml was diluted to 100 ml with distilled water to get the concentration of 50 µg/ ml of Tapentadol Hydrochloride. Further 2 ml was pipetted out in to a series of six 25 ml volumetric flasks, added 1.5 ml of 0.5% w/v solution of ferric chloride and shaken for 2 minutes. Then 3.2 ml of 0.1% w/v solution of potassium ferricyanide was added, shaken well and kept aside for another 2 minutes. To this solution, 2.5 ml of 1M hydrochloric acid was added and allowed to stand for further 2 minutes, and made up to 25 ml with distilled water. The absorbances of solutions were measured at 737 nm. This procedure was repeated for six times.

4.2.3.2.10 Recovery

4.2.3.2.10.1 *Preparation of Tapentadol Hydrochloride raw material stock solution*

An accurately weighed quantity of 400 mg of Tapentadol Hydrochloride was transferred into 10 ml volumetric flask, added sufficient distilled water to dissolve the substance and made up to the volume to 10 ml with distilled water. The solution contains 40 mg/ ml of Tapentadol Hydrochloride.

4.2.3.2.10.2 Procedure

The recovery experiment was done by adding known concentrations of raw material stock solution of Tapentadol Hydrochloride to the preanalyzed formulation. The tablet powder equivalent to 100 mg of Tapentadol Hydrochloride was weighed in to three separate 100 ml volumetric flasks. To this added 2 ml, 2.5 ml and 3 ml of Tapentadol Hydrochloride raw material stock solution. Added about 80 ml of distilled water and sonicated for 15 minutes. The solution was made up to 100 ml with distilled water. The solution was centrifuged for 15 minutes at 2000 rpm and the solution was filtered through a Whatmann filter paper No.41. From the clear solution, 5 ml of the clear solution was transferred in to a series of 100 ml volumetric flasks. Further 2 ml was pipetted out in to a series of 25 ml volumetric flasks, added 1.5 ml of 0.5% w/v solution of ferric chloride and shaken for 2 minutes. Then 3.2 ml of 0.1% w/v solution of potassium ferricyanide was added, shaken well and kept aside for another 2 minutes. To this solution, 2.5 ml of 1M hydrochloric acid was added and allowed to stand for further 2 minutes, and made up to 25 ml with distilled water. The absorbances of solutions were measured at 737 nm. This procedure was repeated for three times.

4.2.3.2.11 Validation of developed method

4.2.3.2.11.1 *Linearity*

A calibration curve was plotted between concentration and amplitude. Tapentadol Hydrochloride was linear with the concentration range of 1 - 10 µg/ ml.

4.2.3.2.11.2 *Precision*

The repeatability of the method was confirmed by the analysis of formulation was repeated for six times with the same concentration. The amount of drug present in the tablet formulation was calculated. The percentage RSD and confidence interval were calculated.

The intermediate precision of the method was confirmed by intraday and inter day analysis i.e. the analysis of formulation was repeated three times in the same day and on three successive days. The amount of drugs was determined. The percentage RSD and confidence interval were calculated.

4.2.3.2.11.3 *Accuracy*

Accuracy of the method was confirmed by recovery studies. To the pre-analyzed formulation, a known quantity of raw material of Tapentadol Hydrochloride was added and the procedure was followed as per the analysis of formulation. The amount of each drug recovered was calculated. This procedure was repeated for three times for each concentration. The % RSD and confidence interval were calculated.

4.2.3.2.11.4 *Ruggedness*

Ruggedness of the method was confirmed by the analysis of formulation was done by using different instruments and different analysts. The amount was calculated. The % RSD and confidence interval were calculated.

4.2.3.2.11.5 *LOD and LOQ*

The limit of detection (LOD) is the lowest concentration at which the results still satisfy some predetermined acceptance criteria. Below the LOD, the results fail to meet these criteria (analysis is not feasible). It may expressed as

$$LOD = \frac{3.3\,\sigma}{S}$$

Where, σ = the standard deviation of the response

S = the slope of the calibration curve

The limit of quantitation (LOQ) is set at a higher concentration than LOD, in the statistical method, it is 10 SD above the mean blank value, this presenting a greater probability that a value at the LOQ is real and not just a random fluctuation of the blank reading.

$$LOQ = \frac{10\,\sigma}{S}$$

Where, σ = the standard deviation of the response

S = the slope of the calibration curve.

4.2.4 HPTLC Method

In HPTLC, the separation of the components of a mixture is based upon the principle of adsorption. HPTLC is differ from the TLC in the size of the silica gel used as the stationary phase, automated sampling application and detection. In the present study, a twin trough chamber and silica gel 60 F 254 plates were used.

4.2.4.1 Optimization of chromatographic method

The initial separation conditions used for the separation of Tapentadol Hydrochloride as follows.

Stationary Phase	-	Silica gel 60 F 254 HPTLC Plates
Mobile Phase	-	Methanol : Ethyl acetate : Hexane
Ratio	-	8 : 1 : 1 % v/v/v
Detection	-	UV light
Temperature	-	Room temperature
Chamber	-	Camag Twin Trough Chamber
Development Mode	-	Ascending Mode

The mobile phase was allowed to saturate for 20 minutes in the chamber. A 1mg/ ml solution was spotted on the plates and developed inside the saturated chamber.

4.2.4.2 Selection of Mobile Phase

Different mixtures of mobile phase were tried to choose the best mobile phase for the analysis. They include the following

n-butanol: Methanol :Ammonia (5:4:1 % v/v/v)

Ethanol: Ethyl Acetate: Water (6:3:1 % v/v/v)

Ethanol: Hexane: Water (7:1:2 % v/v/v)

Ethanol: Toluene: Water (7:1:2 % v/v/v)

n-butanol: Methanol: Ammonia (5:4:1 % v/v/v)

n-butanol: Methanol: Ammonia (3:6:1 % v/v/v)

n-butanol: Methanol: Ammonia (6:3:1 % v/v/v)

n-butanol: Methanol: Ammonia (7:2:1 % v/v/v)

Methanol: Ethyl acetate: Water (9:0.5:0.5 % v/v/v)

Methanol: Ethyl acetate: Water (8:1:1 % v/v/v)

Methanol: Ethyl acetate: Water (7:2:1 % v/v/v)

Methanol: Ethyl acetate: Water (6:3:1 % v/v/v)

From the above list of mobile phase the mobile phase Methanol: Ethyl acetate: Water (8:1:1 % v/v/v) was found to be a better mobile phase. The drug was eluted with good peak.

4.2.4.3 Selection of detection wavelength

The spectrum of Tapentadol Hydrochloride was recorded in the selected mobile phase. 270 nm was selected as detection wave length (λ max, of Taapentadol Hydrochloride in Mobile phase) where the absorbance was maximum.

4.2.4.4 Optimized Chromatographic Conditions

After conforming with the mobile phase and the detection wavelength, the optimised conditions for the method was as follows,

Stationary Phase	-	Silica Gel 60 F 254 HPTLC Plates
Mobile Phase	-	Methanol: Ethyl acetate: Water
Ratio	-	(8:1:1 % v/v/v)
Detection	-	CAMAG TLC Scanner 3, at 270 nm

Temperature	-	Room Temperature
Chamber	-	CAMAG Twin Trough Chamber
Development Mode	-	Ascending Mode

4.2.4.5 Preparation of standard stock solution

10 mg of Tapentadol Hydrochloride was weighed accurately and transferred into a 10 ml volumetric flask. Dissolved in methanol and the volume was made up to 10 ml with methanol to get a concentration of 1mg / ml.

4.2.4.6 Preparation of working standard solution

From the stock solution, 1 ml was diluted to 10 ml with methanol. The solution contains 100 µg/ ml.

4.2.4.7 Linearity and Calibration Curve

Form the working standard solution, 1 - 6 µl were spotted at regular intervals in the plate to get a concentration range of 100 to 600 ng/ µl of Tapentadol Hydrochloride. The plates were developed and the calibration graph was plotted using peak area versus concentration.

4.2.4.8 Quantification of formulation

Twenty tablets were weighed accurately and the average weight of each tablet was determined. The tablets were crushed into fine powder. The tablet powder equivalent to 10 mg of Tapentadol Hydrochloride was weighed in to a 10 ml volumetric flask, dissolved in methanol and sonicated for 15 minutes and made up to 10 ml with methanol. This solution was centrifuged for 15 minutes at 2000 rpm. The solution was filtered through a Whatmann filter paper No. 41. From this solution, 1 ml was diluted to 10 ml with methanol to get 100 ng/ µl of Tapentadol Hydrochloride. Six 3 µl spots were placed on the plates and the chromatograms were recorded. From the peak area the amount of the drug was calculated.

4.2.4.9 Recovery studies

4.2.4.9.1 *Preparation of Tapentadol Hydrochloride raw material stock solution*

40 mg of Tapentadol Hydrochloride raw material was weighed in to a 10 ml volumetric flask, dissolved well with methanol and made up to 10 ml with methanol to get the concentration of 4 mg/ ml.

4.2.4.9.2 *Procedure*

The recovery experiment was done by adding known concentrations of raw material stock solution of Tapentadol Hydrochloride to the preanalyzed formulation. Tablet powder equivalent to 10 mg of Tapentadol Hydrochloride was weighed in to three separate 10 ml volumetric flasks and dissolved in methanol, to this added 2 ml, 2.5 ml and 3 ml of Tapentadol Hydrochloride raw material stock solution. Sonicated for 15 minutes and made up to 10 ml with methanol. This solution was centrifuged for 15 minutes at 2000 rpm. The solution was filtered through a Whatmann filter paper No. 41. From the clear solution, 1 ml was diluted to 10 ml with methanol. From this solution, 3 µl spot was placed from each flask and the chromatograms were recorded. The amount of drug recovered was calculated. The procedure was reported for three times.

4.2.4.10 Validation of developed method

4.2.4.10.1 *Linearity*

A calibration curve was plotted between concentration and amplitude. Tapentadol Hydrochloride was linear with the concentration range of 100 - 600 ng/ µl.

4.2.4.10.2 *Precision*

The repeatability of the method was confirmed by the analysis of formulation was repeated for six times with the same concentration. The amount of drug present in

the tablet formulation was calculated. The percentage RSD and confidence interval were calculated.

The intermediate precision of the method was confirmed by intraday and inter day analysis i.e. the analysis of formulation was repeated three times in the same day and on three successive days. The amount of drugs was determined. The percentage RSD and confidence interval were calculated.

4.2.4.10.3 ***Accuracy***

Accuracy of the method was confirmed by recovery studies. To the pre-analyzed formulation, a known quantity of raw material of Tapentadol Hydrochloride was added and the procedure was followed as per the analysis of formulation. The amount of each drug recovered was calculated. This procedure was repeated for three times for each concentration. The % RSD and confidence interval were calculated.

4.2.4.10.4 ***LOD and LOQ***

The limit of detection (LOD) is the lowest concentration at which the results still satisfy some predetermined acceptance criteria. Below the LOD, the results fail to meet these criteria (analysis is not feasible). It may expressed as

$$LOD = \frac{3.3\ \sigma}{S}$$

Where, σ = the standard deviation of the response

S = the slope of the calibration curve

The limit of quantitation (LOQ) is set at a higher concentration than LOD, in the statistical method, it is 10 SD above the mean blank value, this presenting a greater probability that a value at the LOQ is real and not just a random fluctuation of the blank reading.

$$LOQ = \frac{10\,\sigma}{S}$$

Where, σ = the standard deviation of the response

S = the slope of the calibration curve.

Results

&

Discussion

5. RESULTS AND DISCUSSION

The following methods were developed and validated for the estimation of Tapentadol Hydrochloride in pure and in tablet dosage form. The methods are

1) UV spectroscopic method
2) Difference spectrophotometric method
3) Visible spectrophotometric method
    a) Method – I (using MBTH reagent)
    b) Method – II (using potassium ferricyanide)
4) HPTLC method

5.1. UV SPECTROSCOPIC METHOD

Tapentadol Hydrochloride was obtained as a gift sample from MSN Laboratories Limited, Hyderabad and was identified by melting point analysis and IR spectral studies (figure 1).

The solubility of Tapentadol Hydrochloride was determined as per Indian pharmacopoeia (table -1). The number of polar and non - polar solvents were tried to dissolve the drug. From the solubility data Tapentadol Hydrochloride is very soluble in Distilled water, 0.1M Hydrochloric Acid, pH-3.0 (Acid Phthalate Buffer), pH-5.0 (Neutralized Phthalate Buffer), pH-7.0 (Phosphate Buffer), pH-9 (Alkaline Borate Buffer); freely soluble in Methanol, Ethanol, Di Methyl Formamide, Acetic Acid, Pyridine; soluble in n-Butanol; sparingly soluble in 0.1M Sodium hydroxide, Iso Propyl Alcohol; slightly soluble in Acetonitrile, Ethyl Acetate, Dichloromethane, Toluene and practically in soluble in Acetone, Chloroform, Benzene, Diethyl ether. From the solubility profile, Distilled water, 0.1M Hydrochloric acid, pH-3.0 (Acid Phthalate Buffer), pH-5.0 (Neutralized Phthalate Buffer), pH-7.0 (Phosphate Buffer), pH-9 (Alkaline Borate Buffer) can be used as solvents. But when compared to distilled water,

the other solvents to be prepared before using. Preparations of such solvents are time consuming. Even though the cut off wave length for distilled water is very low (205 nm) when compared to other solvents. Hence distilled water was selected as solvent for the analysis of Tapentadol Hydrochloride.

The spectrum of Tapentadol Hydrochloride was recorded in the wavelength range of 200 – 400 nm. From the spectrum, the maximum absorbance was observed at 272.5 nm. Hence 272.5 nm was selected for the analysis of Tapentadol Hydrochloride. This is shown figure 2. The stability of Tapentadol Hydrochloride was studied by measuring the absorbance at different time intervals. It was observed that the drug was stable up to 24 hours in distilled water.

Different aliquots of Tapentadol Hydrochloride were prepared in the concentration range of 10 to 60 µg/ ml. The absorbances of solutions were measured at 272.5 nm. The calibration curve was plotted using concentration against absorbance. The preparation of calibration curve was repeated for six times. The optical characteristics like Correlation coefficient, Slope, Intercept, Molar absorptivity, Sandell's sensitivity, LOD, LOQ and Residual sum of squares were calculated. The correlation coefficient value for the calibration graph was found to be 0.9999. This indicates that the absorbance were linear with the concentration range of 10 – 60 µg/ ml. The calibration curve of Tapentadol Hydrochloride is shown in figure 3. The optical characteristics are listed in table 2.

To confirm the developed method, quantification of raw material was done and the amount was calculated. The percentage amount of Tapentadol Hydrochloride was found to be 98.21% ± 0.6159. The amount found by this method was close to 100%. Hence this method can be applied for analysis of formulation. The results are shown in table 3.

Tapol-100 tablets (MSN Laboratories Limited, Hyderabad) were selected for analysis. The nominal concentration of Tapentadol Hydrochloride from the linearity was prepared and the absorbance of the solution was measured at 272.5 nm. The amount of test solution was calculated by using slope and intercept values. This procedure was repeated for six times to ensure the precision of the method. The percentage label claim in tablet formulation was found to be 100.56 ± 0.3153. The amount present in tablet formulation was good concord with the label claim and the Percentage Relative Standard Deviation (% RSD) value was found to be 0.3136. The low % RSD value indicates that the method has good precision. The results of analysis are shown in table 4.

The intermediate precision of the method was confirmed by Intraday and Inter day analysis. The analysis of formulation was repeated for three times in the same day and one time in the three successive days. The percentage RSD value for intraday and inter day analysis of Tapentadol Hydrochloride was found to be 0.2781 and 0.3495, respectively. The reports of analysis are shown in tables 5 and 6. The results showed that the intermediate precision of the method was confirmed.

The developed method was validated for Ruggedness. It refers to the analysis should be done in same laboratory which may include multiple analysts, multiple instruments and different sources of the reagents and so on. In the present work, it was confirmed by different analysts and different instrument. The percentage RSD value for analyst 1 and analyst 2 were found to be 0.3669 and 0.3980, respectively. The % RSD value for instrument 1 and instrument 2 were found to be 0.3538 and for 0.8992, respectively. The low % RSD values indicate that the method was more rugged. Reports of analysis are shown in tables 7 and 8 for different analyst and different instruments, respectively.

The accuracy of the method was confirmed by recovery studies. To the pre-analyzed formulation a known quantity of Tapentadol Hydrochloride raw material solution was added at three different concentrations. The concentration of standard raw material added were 80%, 100% and 120% of the sample concentration. The absorbances of the solutions were measured and the percentage recovery was calculated. The percentage recovery was found to be in the range of 100.46% to 100.63% of Tapentadol Hydrochloride. The % RSD was found to be less than 2. The low % RSD values indicated that there are no interferences due to excipients used in formulation during the analysis of Tapentadol Hydrochloride from tablets formulation. Hence the method was found to be accurate. The recovery data are shown in table 9.

5. 2 DIFFERENCE SPECTROSCOPIC METHOD

This method is based on the difference in absorbance measurements between the absorbance maxima and minima. It is a measure of amplitude between maxima and minima of two equimolar solutions of the Tapentadol Hydrochloride in different chemical forms, which exhibits different spectral characteristics.

Distilled water, 0.1M sodium hydroxide and 0.1 M hydrochloric acid were selected as solvents for difference spectroscopic method because of its easy availability, difference in pH, and high stability of Tapentadol Hydrochloride in all the solvents.

The stock solution was prepared in distilled water. Then the difference spectrum was recorded by placing same concentration of further diluted Tapentadol Hydrochloride in 0.1M sodium hydroxide and 0.1M hydrochloric acid in sample and reference cell, respectively. The difference spectra of Tapentadol Hydrochloride were recorded as shown in figure 4. From the spectrum, Tapentadol Hydrochloride showed two maximas at 238 nm and 290 nm and one minima at 269.5 nm. In the maxima at 238 nm, the interference due to solvent was obtained. Hence 290 nm was selected as the

maxima and 269.5 nm was selected as minima. The measured value was the amplitude between maxima and minima of two equimolar solutions in different chemical forms, which exhibits different spectral characteristics. Hence these wavelengths were selected for analysis of Tapentadol Hydrochloride by difference spectrophotometric method.

Serial dilutions of Tapentadol Hydrochloride were prepared in the concentrations range of 3-18 µg/ml in both 0.1M sodium hydroxide and 0.1M hydrochloric acid. The amplitude was measured the maxima at 290 nm and minima at 269.5 nm. The calibration curve was plotted using concentration versus amplitude. The procedure was repeated for six times. The optical parameters like correlation co-efficient, Slope, Intercept, Sandells sensitivity, Molar absorptivity, LOD, LOQ and Residual Sum of Square were calculated. These are shown in Table 10. The correlation co-efficient value was found to be 0.9999 indicates that the observed amplitude was linear with selected concentrations of Tapentadol Hydrochloride. The calibration graph is shown in figure 5.

To confirm the developed method, quantification of raw material was done and the amount was calculated. The percentage amount of Tapentadol Hydrochloride was found to be 100.84% ± 0.2562. The amount found by this method was close to 100%. Hence this method was applied for analysis of formulation. The results are shown in table 11.

Tapol-100 (MSN Laboratories Limited, Hyderabad) containing 100 mg of Tapentadol was selected for analysis. The nominal concentration of Tapentadol Hydrochloride from the linearity was prepared and the amplitude was measured at maxima at 290 nm and minima at 269.5 nm. The percentage of Tapentadol Hydrochloride present in tablet formulation was found to be 100.60 ± 0.5872. The amount present in tablet formulation was good concord with the label claim and the

Percentage Relative Standard Deviation (% RSD) value was found to be 0.5837. The low % RSD value indicates that the method has good precision. The results of analysis are shown in table 12.

The intermediate precision of the method was confirmed by Intraday and Inter day analysis. The analysis of formulation was repeated for three times in the same day and one time in the three successive days. The percentage RSD value for intraday and inter day analysis of Tapentadol Hydrochloride was found to be 0.3188 and 0.2667, respectively. The reports of analysis are shown in tables 13 and 14. The low % RSD values showing the intermediate precision of the method was confirmed.

The developed method was validated for Ruggedness. It refers to the analysis should be done in same laboratory which may include multiple analysts, multiple instruments and different sources of the reagents and so on. In the present work, it was confirmed by different analysts and different instrument. The percentage RSD value for analyst 1 and analyst 2 was found to be 0.2364 and 0.4066, respectively. The % RSD value for instrument 1 and instrument 2 were found to be 0.7191 and 0.4620, respectively. The low % RSD values indicate that the method was more rugged. Reports of analysis are shown in tables 15 and 16 for different analysts and different instruments, respectively.

The accuracy of the method was confirmed by recovery studies. To the pre-analyzed formulation a known quantity of Tapentadol Hydrochloride raw material solution was added at three different concentrations. The concentrations of standard were 80%, 100% and 120% of the sample concentration. The absorbances of the solutions were measured and the percentage recovery was calculated. The percentage recovery was found to be in the range of 98.37% to 99.04% of Tapentadol Hydrochloride. The % RSD was found to be less than 2. The low % RSD values

indicated that there are no interferences due to excipients used in formulation during the analysis of Tapentadol Hydrochloride from tablets formulation. Hence the method was found to be accurate. The recovery data are shown in table 17.

5.3 VISIBLE SPECTROPHOTOMETRIC METHOD

5.3.1 Colorimetric Method - I

Tapentadol Hydrochloride has a phenolic hydroxyl group which is on treatment with MBTH reagent (3-Methyl-2-benzothiazolinone hydrazone hydrochloride monohydrate) in the presence of oxidizing agent (ceric ammonium sulphate) give a stable orange red coloured chromogen. The measurement of the intensity of coloured chromogen is the basis of this method.

A solution of 10 µg/ ml was prepared from the stock solution and added 0.8ml of 0.1% w/v MBTH reagent and 1ml of 0.01M ceric ammonium sulphate was added with shaking, the solution was made up to the volume with distilled water. The intensity of the coloured solution was scanned in the visible region of 400 - 800 nm. The spectrum was recorded. The recorded spectra showed that at 514 nm Tapentadol Hydrochloride has the maximum absorbance. Hence this was selected as an analytical wavelength. This is shown in figure 6. The optimizations of the reagents were done by measuring the absorbance of drug solution by adding different concentrations and volumes of ceric ammonium sulphate and MBTH reagent. The absorbance was plotted against different concentrations of ceric ammonium sulphate, different volumes of ceric ammonium sulphate and different concentrations of MBTH reagent, different volumes of MBTH reagent. These are shown in figures 7, 8, 9 and 10, respectively.

From the study it was observed that addition of 1 ml of 0.01M ceric ammonium sulphate and 0.8 ml of 0.1% w/v MBTH reagent showed the maximum colour intensity.

Hence these are selected as the optimized concentrations and volumes of ceric ammonium sulphate and MBTH.

Different aliquots of Tapentadol Hydrochloride were prepared in the concentration range of 2 to 24 µg/ ml. The absorbances of solutions were measured at 514 nm. The calibration curve was plotted using concentration against absorbance. The preparation of calibration curve was repeated for six times. The optical characteristics like Correlation coefficient, Slope, Intercept, Molar absorptivity, Sandell's sensitivity, LOD, LOQ and Residual Sum of Squares were calculated. The correlation coefficient value for the calibration graph was found to be 0.9991. This indicates that the absorbance were linear with the concentration range of 2 - 24 µg/ ml. The calibration curve of Tapentadol Hydrochloride is shown in figure 11. The optical characteristics are listed in table 18.

To confirm the developed method, quantification of raw material was done and the amount was calculated. The percentage amount of Tapentadol Hydrochloride was found to be 101.08% ± 0.7682. The amount found by this method was close to 100%. Hence this method can be applied for the analysis of formulation. The results are shown in table 19.

Tapol-100 tablets (MSN Laboratories Limited, Hyderabad), containing 100 mg of Tapentadol Hydrochloride) were selected for analysis. The nominal concentration of Tapentadol Hydrochloride from the linearity was prepared and the absorbance of the solution was measured at 514 nm. The amount of test solution was calculated by using slope and intercept valves. This procedure was repeated for six times to ensure the precision of the method. The percentage label claim in tablet formulation was found to be 100.05% ± 0.3318. The amount present in tablet formulation was good concord with the label claim and the Percentage Relative Standard Deviation (% RSD) value was

found to be 0.3317. The low % RSD value indicates that the method has good precision. The results of analysis are shown in table 20.

The intermediate precision of the method was confirmed by Intraday and Inter day analysis. The analysis of formulation was repeated for three times in the same day and one time in the three successive days. The percentage RSD value for intraday and inter day analysis of Tapentadol Hydrochloride was found to be 0.4819 and 0.2227, respectively. The reports of analysis are shown in tables 21 and 22, respectively. The results showed that the intermediate precision of the method was confirmed.

The developed method was validated for Ruggedness. It refers to the analysis should be done in same laboratory which may include multiple analysts, multiple instruments and different sources of the reagents and so on. In the present work, it was confirmed by different analysts and different instrument. The percentage RSD value for analyst 1 and analyst 2 were found to be 0.1247 and 0.3622, respectively. The % RSD value for instrument 1 and for instrument 2 were found to be 0.3564 and 0.7566, respectively. The low % RSD values indicate that the method was more rugged. Reports of analysis are shown in tables 23 and 24 for different analysts and different instruments, respectively.

The accuracy of the method was confirmed by recovery studies. To the preanalyzed formulation a known quantity of Tapentadol Hydrochloride raw material solution was added at three different concentrations. The concentrations of standard were 80%, 100% and 120% of the sample concentration. The absorbance of the solutions was measured and the percentage recovery was calculated. The percentage recovery was found to be in the range of 101.23% to 101.87% of Tapentadol Hydrochloride. The % RSD values were found to be less than 2. The low % RSD values indicated that there is no interference due to excipients used in formulation during the

analysis of Tapentadol Hydrochloride from tablets formulation. Hence the method was found to be accurate. The recovery data are shown in table 25.

5.3.2 Colorimetric Method - II

Tapentadol Hydrochloride has a phenolic hydroxyl group which is on treatment with ferric chloride and potassium ferricyanide in the presence of acidic medium give a stable Prussian blue coloured chromogen. The measurement of the intensity of coloured chromogen is the basis of this method.

A solution of 4 µg/ ml was prepared from the stock solution and added 1.5 ml of 0.5% w/v ferric chloride reagent with shaking for 2 minutes and 3.2 ml of 0.1% w/v potassium ferricyanide was added, kept aside for 2 minutes and then added 1ml of 1M Hydrochloric acid stand for further 2 minutes, then the solution was made up to the volume with distilled water. The intensity of the coloured solution was scanned in the visible region of 400 - 800 nm. The spectrum was recorded. The recorded spectra showed that at 737 nm Tapentadol Hydrochloride has the maximum absorbance. Hence this was selected as an analytical wavelength. This is shown in figure 12.

The optimizations of the reagents were done by measuring the absorbance of drug solution by adding different concentrations and volumes of ferric chloride, Potassium ferricyanide and hydrochloric acid. The absorbance was plotted against different concentrations of ferric chloride and different volumes of ferric chloride, different concentrations of potassium ferricyanide, different volumes of potassium ferricyanide, and different concentrations of hydrochloric acid, different volumes of hydrochloric acid, and. These are shown in figures 13, 14, 15, 16, 17 and 18, respectively.

From the study it was observed that addition of 1 ml of 0.5% w/v ferric chloride, 3.2 ml of 0.1% w/v potassium ferricyanide and 2.5 ml of 1M hydrochloric acid showed

the maximum colour intensity. Hence these are selected as the optimized concentrations and volumes of ferric chloride, potassium ferricyanide and hydrochloric acid.

Different aliquots of Tapentadol Hydrochloride were prepared in the concentration range of 1 to 10 µg/ ml. The absorbances of solutions were measured at 737 nm. The calibration curve was plotted using concentration against absorbance. The preparation of calibration curve was repeated for six times. The optical characteristics like Correlation coefficient, Slope, Intercept, Molar absorptivity, Sandell's sensitivity, LOD , LOQ and Residual Sum of Square were calculated. The correlation coefficient value for the calibration graph was found to be 0.9997. This indicates that the absorbance were linear with the concentration range of 1- 10 µg/ ml. The calibration curve of Tapentadol Hydrochloride is shown in figure 19. The optical characteristics are listed in table 26.

To confirm the developed method, quantification of raw material was done and the amount was calculated. The percentage amount of Tapentadol Hydrochloride was found to be 100.16% ± 0.2605. The amount found by this method was close to 100%. Hence this method can be applied for the analysis of formulation. The results are shown in table 27.

Tapol-100 tablets (MSN Laboratories Limited, Hyderabad), containing 100 mg of Tapentadol Hydrochloride) were selected for analysis. The nominal concentration of Tapentadol Hydrochloride from the linearity was prepared and the absorbance of the solution was measured at 737 nm. The amount of test solution was calculated by using slope and intercept valves. This procedure was repeated for six times to ensure the precision of the method. The percentage label claim in tablet formulation was found to be 100.13% ± 0.8343. The amount present in tablet formulation was good concord with the label claim and the Percentage Relative Standard Deviation (% RSD) value was

found to be 0.8332. The low % RSD value indicates that the method has good precision. The results of analysis are shown in table 28.

The intermediate precision of the method was confirmed by Intraday and Inter day analysis. The analysis of formulation was repeated for three times in the same day and one time in the three successive days. The percentage RSD value for intraday and inter day analysis was found to be 0.2572 and 0.5914, respectively. The reports of analysis are shown in tables 29 and 30, respectively. The results showed that the intermediate precision of the method was confirmed.

The developed method was validated for Ruggedness. It refers to the analysis should be done in same laboratory which may include multiple analysts, multiple instruments and different sources of the reagents and so on. In the present work, it was confirmed by different analysts and different instrument. The percentage RSD value for analyst 1 and analyst 2 was found to be 0.2819 and 0.7941, respectively. The % RSD value for instrument 1 and instrument 2 were found to be 0.7349 and 0.3157, respectively. The low % RSD values indicate that the method was more rugged. Reports of analysis are shown in tables 31 and 32 for different analysts and different instruments, respectively.

The accuracy of the method was confirmed by recovery studies. To the preanalyzed formulation a known quantity of Tapentadol Hydrochloride raw material solution was added at three different concentrations. The concentrations of standard were 80%, 100% and 120% of the sample concentration. The absorbance of the solutions was measured and the percentage recovery was calculated. The percentage recovery was found to be in the range of 100.13% to 100.75% of Tapentadol Hydrochloride. The % RSD values were found to be less than 2. The low % RSD values indicated that there is no interference due to excipients used in formulation during the

analysis of Tapentadol Hydrochloride from tablets formulation. Hence the method was found to be accurate. The recovery data are shown in table 33.

5.4 HPTLC METHOD

The initial separation was made using various mobile phase using chloroform, methanol, formic acid, acetic acid, toluene, ethyl acetate, ethanol, ammonia and water in different combinations. The mobile phase tried were n-butanol: Methanol :Ammonia (5:4:1 % v/v/v), Ethanol: Ethyl Acetate: Water (6:3:1 % v/v/v), Ethanol: Hexane: Water (7:1:2 % v/v/v), Ethanol: Toluene: Water (7:1:2 % v/v/v), n-butanol: Methanol: Ammonia (5:4:1 % v/v/v), n-butanol: Methanol: Ammonia (3:6:1 % v/v/v), n-butanol: Methanol: Ammonia (6:3:1 % v/v/v), n-butanol: Methanol: Ammonia (7:2:1 % v/v/v), Methanol: Ethyl acetate: Water (9:0.5:0.5 % v/v/v), Methanol: Ethyl acetate: Water (8:1:1 % v/v/v), Methanol: Ethyl acetate: Water (7:2:1 % v/v/v), Methanol: Ethyl acetate: Water (6:3:1 % v/v/v) were tried.

In n-butanol: Methanol :Ammonia (5:4:1 % v/v/v) the drug was separated and the R_f value found to be 0.90. The R_f value of Tapentadol Hydrochloride was beyond the normal values (range from 0.2 to 0.8), (P.D Sethi et al). Hence the different ratios were tried with the same mobile phase. But there was no remarkable improvement. Hence the mobile phase was switched to Ethanol: Ethyl Acetate: Water (6:3:1 % v/v/v). The R_f value was found to be 0.42. In Methanol: Ethyl Acetate: Water (8:1:1 % v/v/v), the improved R_f value was 0.50. The different ratios of mobile phase were tried (Methanol: Ethyl acetate: Water (9:0.5:0.5 % v/v/v), Methanol: Ethyl acetate: Water (8:1:1 % v/v/v), Methanol: Ethyl acetate: Water (7:2:1 % v/v/v), Methanol: Ethyl acetate: Water (6:3:1 % v/v/v)). Methanol: Ethyl acetate: Water in the ratio of 8:1:1 % v/v was selected for the method since, the drug was eluted with good resolution. 270 nm was selected as the detection wavelength for the analysis. The

spectral conformation of the standard Tapentadol Hydrochloride with sample solution Tapentadol Hydrochloride is given in figure 20.

1 mg/ ml stock solution of Tapentadol Hydrochloride was prepared in methanol. From the stock solution concentration range of 100 to 600 ng/ µl of Tapentadol Hydrochloride chromatogram were developed in the Twin trough Chamber. The linearity chromatograms are shown in figure 21 to 26. The calibration graph was plotted with concentration versus peak area and the correlation coefficient was found to be 0.9995 for Tapentadol Hydrochloride. The calibration graph for Tapentadol Hydrochloride was shown in figure 27. The optical characteristics such as the LOD, LOQ, Slope, Intercept, Regression equation and correlation coefficient are given in table 34.

The tablet powder equivalent to 10 mg of Tapentadol Hydrochloride was taken and dissolved in 10 ml of methanol. The solution was sonicated for 15 minutes and made up to the volume with methanol. This solution was centrifuged at 2000 rpm for 15 minutes and the supernatant liquid is filtered through a Whatmann filter paper no 41. This solution was diluted to get a final solution containing 100 ng/ µl of Tapentadol Hydrochloride theoretically. Six spots were made on the plates with 3 µl solution for each spot. The chromatogram was developed and the peak areas were noted. The percentage purity of Tapentadol Hydrochloride was found to be 99.97% ± 0.2196. The %RSD values for Tapentadol Hydrochloride 0.2197. The chromatograms are given in figures 28 – 33. The data is given in table 35.

The intermediate precision of the method was confirmed by Intraday and Inter day analysis. The analysis of formulation was repeated for three times in the same day and one time in the three successive days. The chromatograms are given in figures 34 – 39. The percentage RSD value for intraday and inter day analysis of

Tapentadol Hydrochloride was found to be 0.0551 and 0.0635, respectively. The reports of analysis are shown in tables 36 and 37, respectively. The results showed that the precision of the method was further confirmed.

The accuracy of the method is confirmed by the recovery analysis. To the pre-analyzed formulation, known quantities of the standard drugs were added at three different concentrations. The amount of Tapentadol Hydrochloride recovered was in the range of 99.96% to 100.36%. The %RSD values were found to be less than 2. The low %RSD values indicate that there is no interference of the excipients during the analysis. The peaks of the developed chromatograms are given in figures 40 to 42.The data of recovery analysis are given in table 38.

Summary & Conclusion

6. SUMMARY AND CONCLUSION

Four simple, rapid, precise and accurate UV - Visible spectrophotometric methods and an isocratic HPTLC method were developed and validated for estimation of Tapentadol Hydrochloride in pure form and in tablet dosage form.

The methods employed for the analysis of Tapentadol Hydrochloride were

1) UV spectroscopic method
2) Difference spectrophotometric method
3) Visible spectrophotometric method
    a) Method - I (using MBTH reagent)
    b) Method - II (using potassium ferricyanide)
4) HPTLC method

6.1 UV SPECTROSCOPIC METHOD

From the solubility data the solvent selected for solubility was found to be distilled water. The λmax of Tapentadol Hydrochloride in Distilled water was 272.5 nm.

Tapentadol Hydrochloride was linear with the concentration range of 10 - 60 µg/ ml. The correlation coefficient value for the calibration graph was found to be 0.9999. The amount of Tapentadol Hydrochloride present in the prepared raw material solution was found to be 98.21 ± 0.6159.

Tapol-100 tablets (MSN Laboratories Limited Hyderabad), containing 100 mg of Tapentadol Hydrochloride) were selected for analysis. The percentage label claim present in the tablet formulation was found to be 100.56 ± 0.3153 of Tapentadol Hydrochloride. The precision of the method was confirmed by the repeated analysis of formulation. The %RSD was found to be 0.3136.

Further precision of the method was confirmed by Intraday and Inter day analysis. The percentage RSD value of the intraday and inter day analysis of Tapentadol Hydrochloride was found to be 0.2781 and 0.3495, respectively. The developed method was validated for ruggedness. The percentage RSD value by analyst 1 and analyst 2 were found to be 0.3669 and 0.3980 respectively and by instrument 1 and instrument 2 were found to be 0.3538 and 0.8992 respectively. The low % RSD values indicate that the developed method was more rugged. The accuracy of the method was confirmed by recovery studies. The percentage recovery was found to be in the range of 100.46% to 100.63% of Tapentadol Hydrochloride.

6.2 DIFEERENCE SPECTROSCOPIC METHOD

Difference spectroscopic method is based on measurement of absorbance difference (amplitude) of Tapentadol Hydrochloride at maxima 290 nm and minima 269.5 nm in 0.1M sodium hydroxide and 0.1M hydrochloric acid solutions, respectively. The measured value is the mplitude of maxima and minima between two equimolar solutions of the analyte in difference chemical forms, which exhibits different spectral characteristics.

Tapentadol Hydrochloride was linear with the concentration range of 3 - 18 µg/ ml. The correlation coefficient value for the calibration graph was found to be 0.9999. The amount of Tapentadol Hydrochloride present in the prepared raw material solution was found to be 100.84 ± 0.2562.

Tapol-100 tablets (MSN Laboratories Limited, Hyderabad) containing 100mg of Tapentadol Hydrochloride) were selected for analysis. The percentage label claim present in the tablet formulation was found to be 100.60 ± 0.5872 of Tapentadol Hydrochloride. The precision of the method was confirmed by the repeated analysis of formulation. The %RSD was found to be 0.5837.

Further precision of the method was confirmed by Intraday and Inter day analysis. The percentage RSD value of the intraday and inter day analysis of Tapentadol Hydrochloride was found to be 0.3188 and 0.2667 respectively. The developed method was validated for ruggedness. The percentage RSD value by analyst 1 and analyst 2 were found to be 0.2364 and 0.4066, respectively and by instrument 1 and instrument 2 were found to be 0.7191 and 0.4620, respectively. The low % RSD values indicate that the developed method was more rugged. The accuracy of the method was confirmed by recovery studies. The percentage recovery was found to be in the range of 98.37% to 99.04% of Tapentadol Hydrochloride.

6.3 VISIBLE SPECTROPHOTOMETRIC METHOD

6.3.1 Colorimetric Method – I

Tapentadol Hydrochloride has a phenolic hydroxyl group, which on treatment with MBTH reagent (3-Methyl-2-benzothiazolinone hydrazone hydrochloride monohydrate) in the presence of oxidizing agent give stable orange red coloured chromogen.

After optimizing the different concentrations and volumes of ceric ammonium sulphate and MBTH reagent, 10 µg/ ml Tapentadol Hydrochloride solution is prepared with 0.8ml of 0.7% MBTH reagent and 1ml of ceric ammonium sulphate are added with shaking, and made up to mark with distilled water. This solution was scanned and the recorded spectra showed that the λmax of Tapentadol Hydrochloride is at 514 nm.

Tapentadol Hydrochloride obeyed Beer's law in the concentration range of 2 to 24 µg/ ml. The correlation coefficient value was found to be 0.9991. The amount of Tapentadol Hydrochloride present in the prepared raw material solution was found to be101.08% ± 0.7682.

Tapol-100 tablets (MSN Laboratories Limited, Hyderabad) containing 100mg of Tapentadol Hydrochloride) were selected for analysis. The percentage label claim present in the tablet formulation was found to be 100.05 ± 0.3318 of Tapentadol Hydrochloride. The precision of the method was confirmed by the reported analysis of formulation. The %RSD was found to be 0.3317.

Further precision of the method was confirmed by Intraday and Inter day analysis. The percentage RSD value of the intraday and inter day analysis of Tapentadol Hydrochloride was found to be 0.4819 and 0.2227 respectively. The developed method was validated for ruggedness. The percentage RSD value by analyst 1 and analyst 2 were found to be 0.1247 and 0.3622 respectively and by instrument 1 and instrument 2 were found to be 0.3564 and 0.7566, respectively. The low % RSD values indicate that the developed method was more rugged. The accuracy of the method was confirmed by recovery studies. The percentage recovery was found to be in the range of 101.23% to 101.87% of Tapentadol Hydrochloride.

6.3.2 Colorimetric Method - II

Tapentadol Hydrochloride has a phenolic hydroxyl group, which on treatment with ferric chloride and potassium ferricyanide in the presence of acidic medium (1M hydrochloric acid) give stable Prussian blue colored chromogen.

After optimizing the different concentrations and volumes of ferric chloride, potassium ferricyanide and 1M hydrochloric acid, 10 µg/ ml Tapentadol Hydrochloride solution is prepared with 1.5ml of 0.5% of ferric chloride with 2min shaking, 3.2ml of 0.1% potassium ferricyanide was added, stand for 2min and 2.5ml of 1N hydrochloric acid was added with shaking and kept aside for 2min, and made up to mark with distilled water. This solution was scanned and the recorded spectra showed that the λmax of Tapentadol Hydrochloride is at 737 nm.

Tapentadol Hydrochloride obeyed Beer's law in the concentration range of 1 to 10 μg/ ml. The optical characteristics like Correlation coefficient, Slope, Intercept, Molar absorptivity, Sandell's sensitivity, LOD and LOQ were calculated. The correlation coefficient value was found to be 0.9997. The amount of Tapentadol Hydrochloride present in the prepared raw material solution was found to be 100.16% ± 0.2605.

Tapol-100 tablets (MSN Laboratories Limited, Hyderabad) containing 100 mg of Tapentadol Hydrochloride) were selected for analysis. The percentage label claim present in the tablet formulation was found to be 100.13 ± 0.8343 of Tapentadol Hydrochloride. The precision of the method was confirmed by the reported analysis of formulation. The %RSD was found to be 0.8332.

Further precision of the method was confirmed by Intraday and Inter day analysis. The percentage RSD value of the intraday and inter day analysis of Tapentadol Hydrochloride was found to be 0.2572 and 0.5914 respectively. The developed method was validated for ruggedness. The percentage RSD value by analyst 1 and analyst 2 were found to be 0.2819 and 0.7941 respectively and by instrument 1 and instrument 2 were found to be 0.7349 and 0.3157. The low % RSD values indicate that the developed method was more rugged. The accuracy of the method was confirmed by recovery studies. The percentage recovery was found to be in the range of 100.13% to 100.75% of Tapentadol Hydrochloride.

6.4 HPTLC METHOD

A simple and rapid HPTLC method was developed for Tapentadol Hydrochloride in bulk and in tablet dosage form. The mobile phase consisting of Methanol: Ethyl acetate: Water in the ratio of 8:1:1% v/v/v was selected for the analysis. From the spectral characteristics, 270 nm was selected as the detection

wavelength for the analysis. With the optimised conditions, the linearity range was fixed as 100 to 600 ng/ µl for Tapentadol Hydrochloride. The correlation coefficient for the Tapentadol Hydrochloride was found to be 0.9995.

Tapol-100 tablets (MSN Laboratories Limited, Hyderabad) containing 100 mg of Tapentadol Hydrochloride) were selected for analysis. The percentage label claim present in the tablet formulation was found to be 99.97 ± 0.2196 of Tapentadol Hydrochloride. The precision of the method was confirmed by the reported analysis of formulation. The %RSD was found to be 0.8332.

Further precision of the method was confirmed by Intraday and Inter day analysis. The percentage RSD value of the intraday and inter day analysis of Tapentadol Hydrochloride was found to be 0.0551 and 0.0635 respectively.

The accuracy of the method was confirmed by recovery studies. The percentage recovery was found to be in the range of 99.96% to 100.36% of Tapentadol Hydrochloride.

Four simple, precise, rapid and accurate UV-Visible spectrophotometric methods and HPTLC method were developed for the determination of Tapentadol Hydrochloride in bulk and in tablet formulation.

When comparing all the five methods HPTLC method is very sensitive, because it is having low LOD and LOQ values. But remaining methods are very economic when compared to HPTLC, because the solvents and instruments used in HPTLC method are very costly. In UV-visible spectrophotometric methods, UV method with a simple solvent (Distilled water) is very less time consuming and economical method. But the linearity range is high because of low absorptivity.

In difference spectrophotometric method difficult to handle both the acidic and basic solutions containing drug at the time of preparation, dilutions, at the time of collecting the reading and it is time consuming method.

Two colorimetric methods are forming well defined colours. But these methods also time consuming methods, because daily we have to prepare each and every reagent and they also ready to react so we need to prevent the contamination at the time of pipetting. At the same time cost of reagents also high.

In HPTLC method preparation of mobile phase and the dilutions of the drug sample is very easy. When comparing with LOD and LOQ values of other methods HPTLC method have high sensitive in quantitative and qualitative analysis. But the cost of instrument and solvents used are very expensive.

Although, the developed methods were validated according to ICH guide lines, and the results obtained in the validation (Accuracy, Precision, Repeatability, Intermediate Precision, Specificity, Detection Limit, Quantitation Limit, Linearity and Range) are within the limits according to ICH guide lines. Hence, it is suggested that the proposed UV-Visible spectrophotometric methods and HPTLC method can be effectively applied for the routine quality control analysis of Tapentadol Hydrochloride in bulk and in its tablet dosage form.

Figures

FIGURE – 1

IR SPECTRUM OF
TAPENTADOL HYDROCHLORIDE

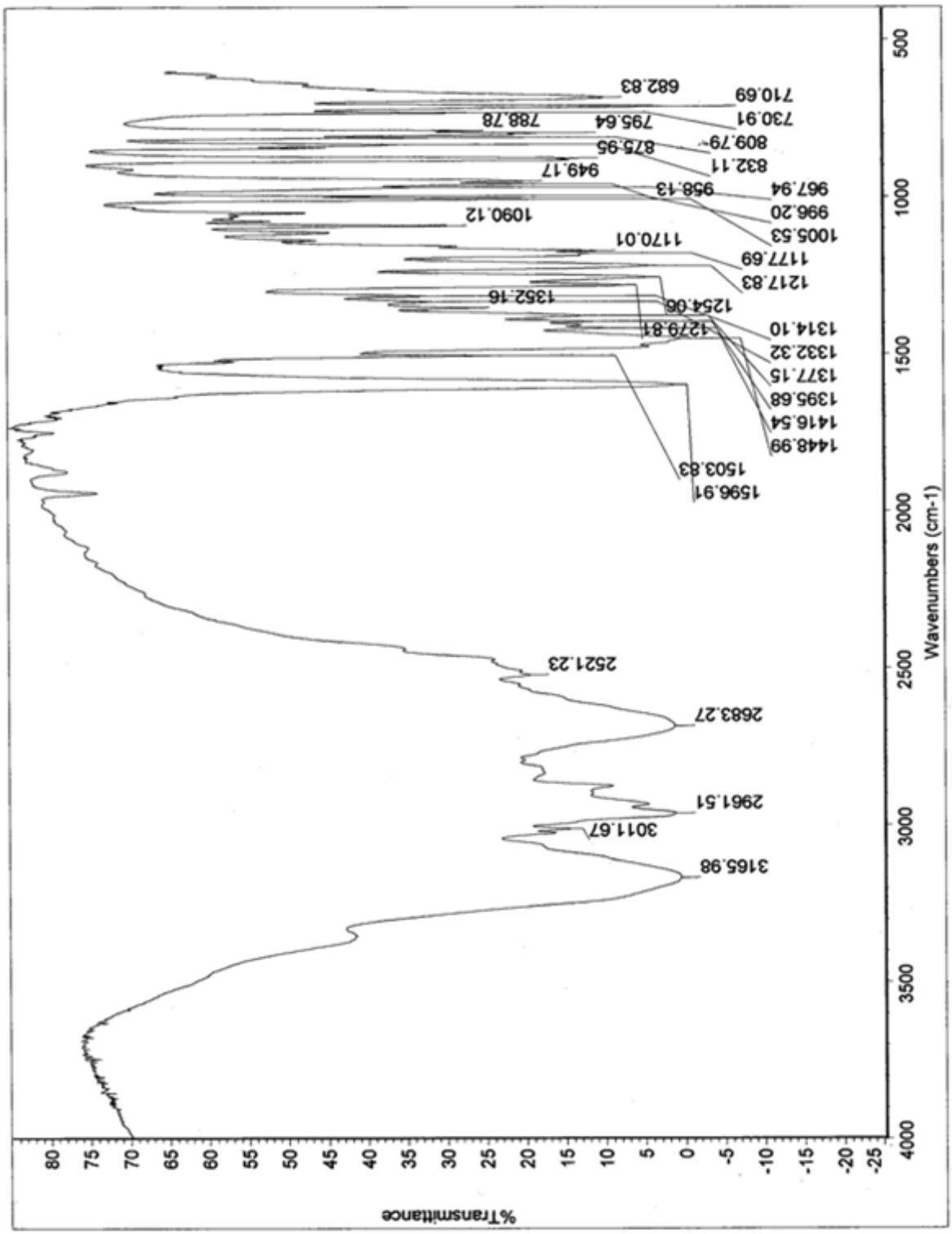


FIGURE - 2

UV SPECTRUM OF TAPENTADOL HYDROCHLORIDE IN DISTILLED WATER (10 µg/ ml)

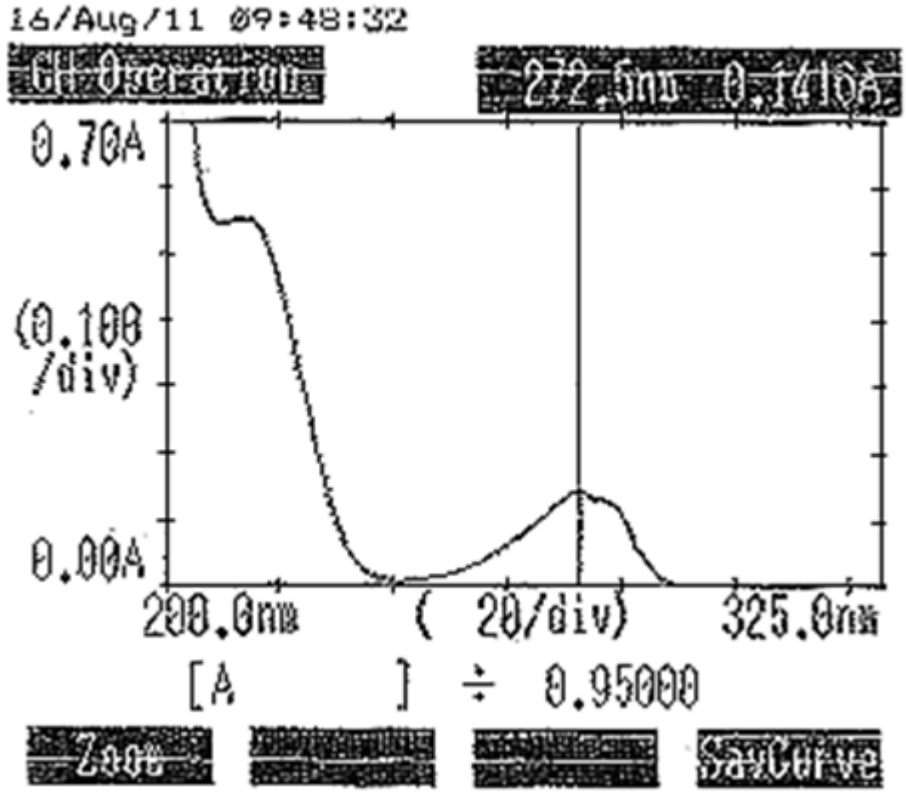


FIGURE - 3

CALIBRATION CURVE OF TAPENTADOL HYDROCHLORIDE IN DISTILLED WATER AT 272.5 nm

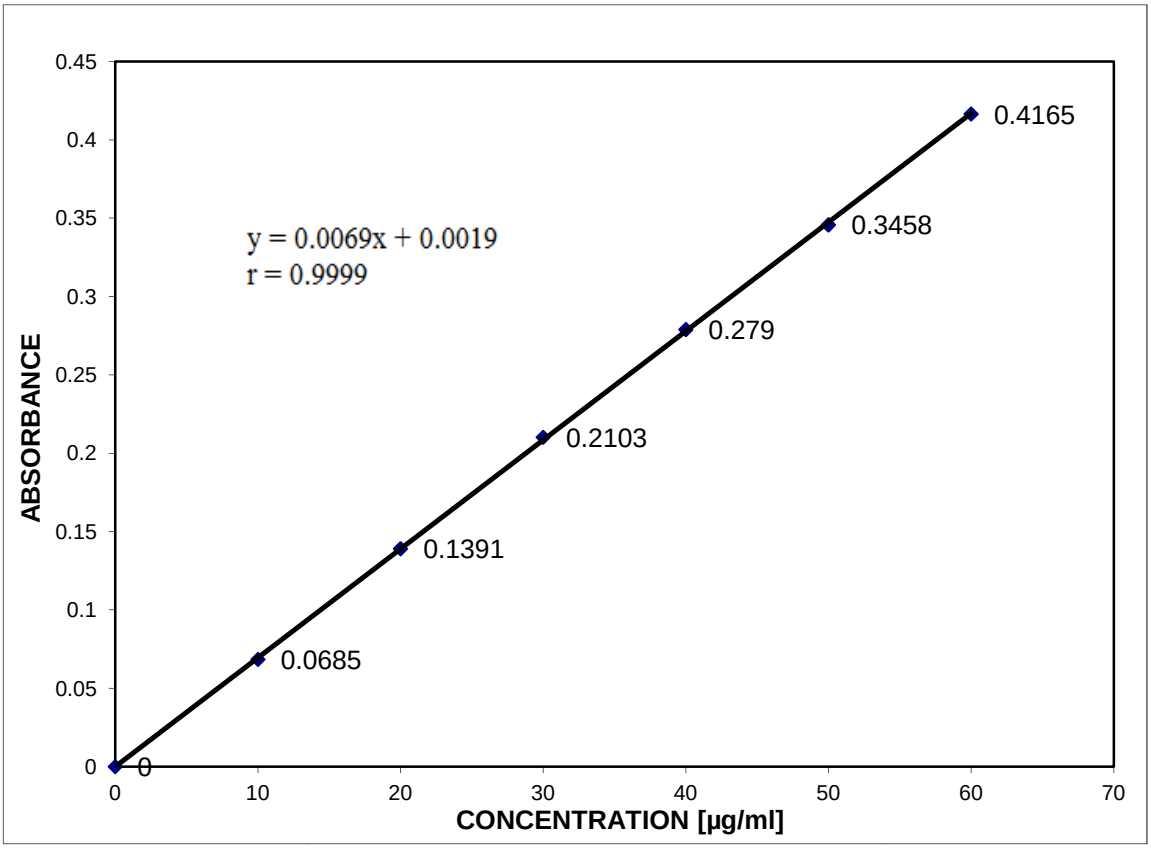
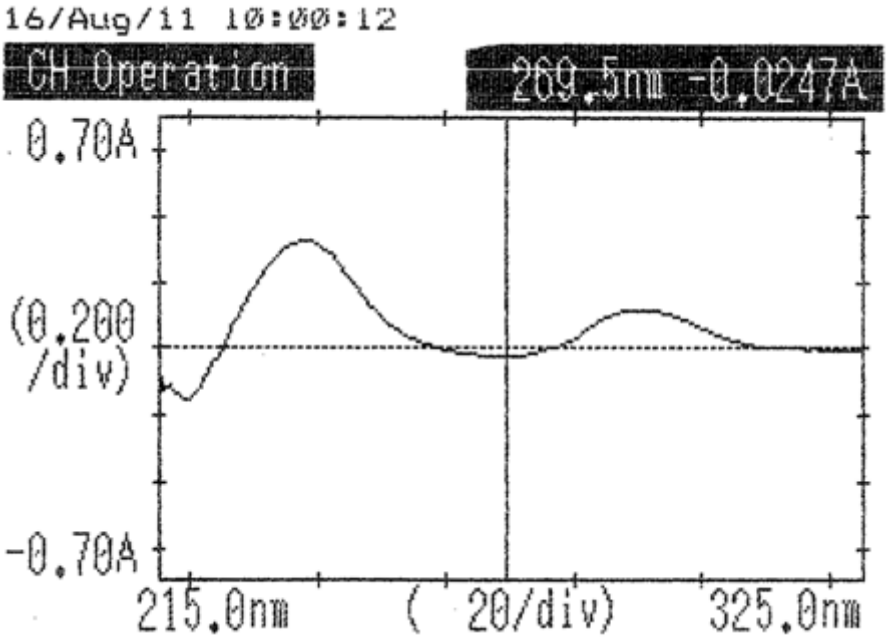


FIGURE - 4

DIFFERENCE SPECTRA OF TAPENTADOL HYDROCHLORIDE (10 µg/ ml)



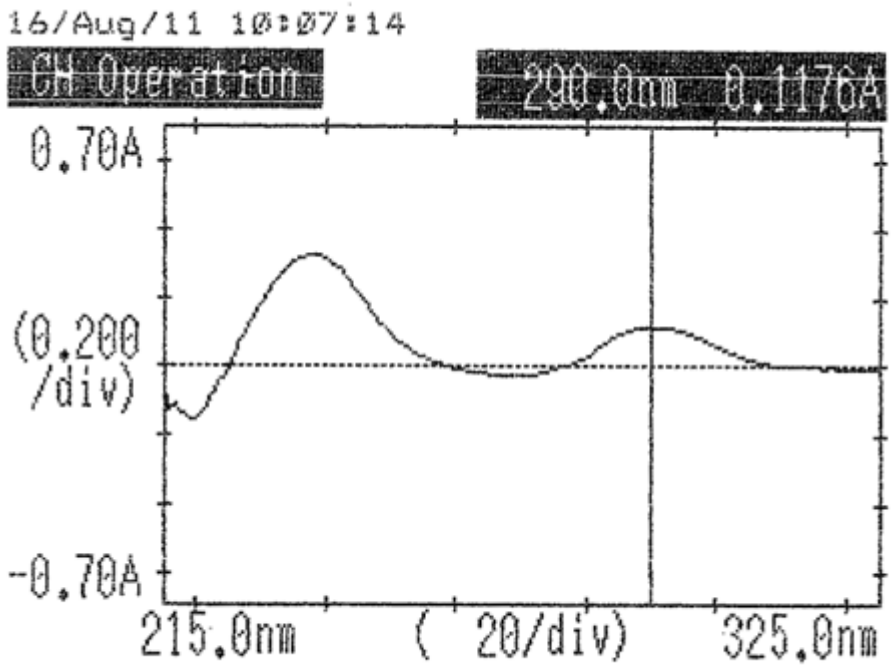


FIGURE - 5

CALIBRATION CURVE OF TAPENTADOL HYDROCHLORIDE BY DIFFERENCE SPECTROPHOTOMETRY

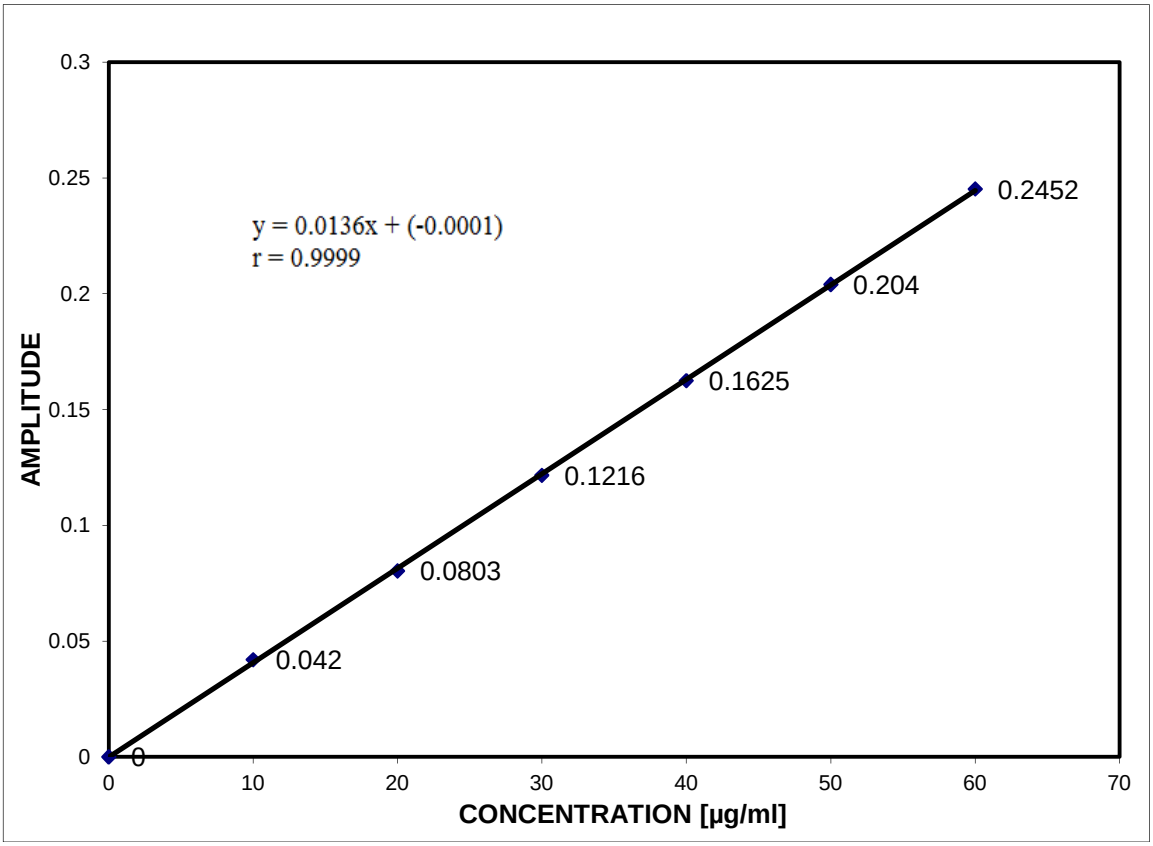


FIGURE – 6

VISIBLE SPECTRUM OF TAPENTADOL HYDROCHLORIDE BY COLORIMETRIC METHOD-I

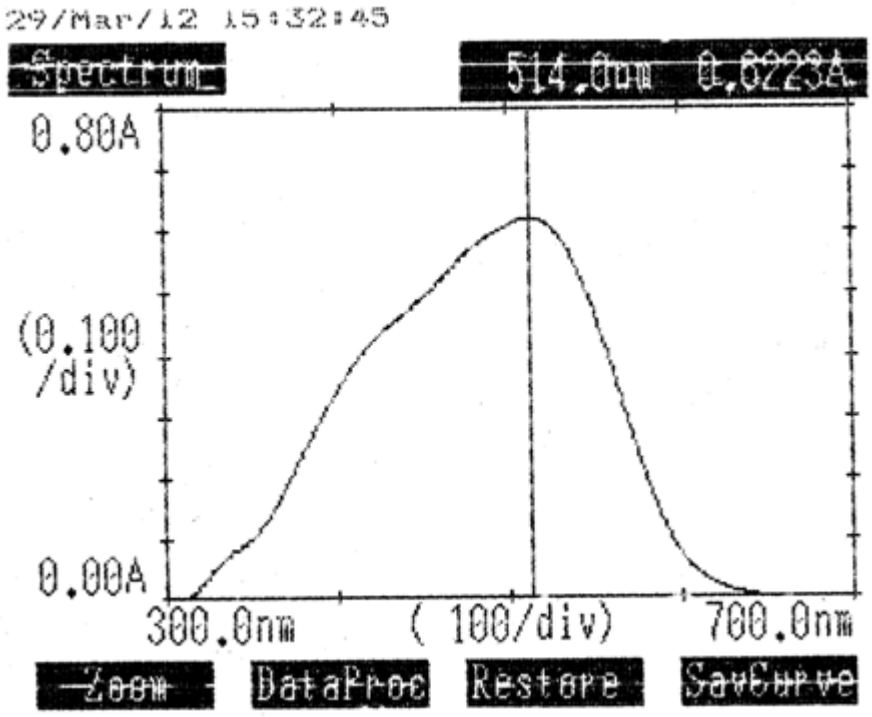


FIGURE-7

OPTIMISATION OF CERIC AMMONIUM SULPHATE BY USING DIFFERENT MOLAR SOLUTIONS

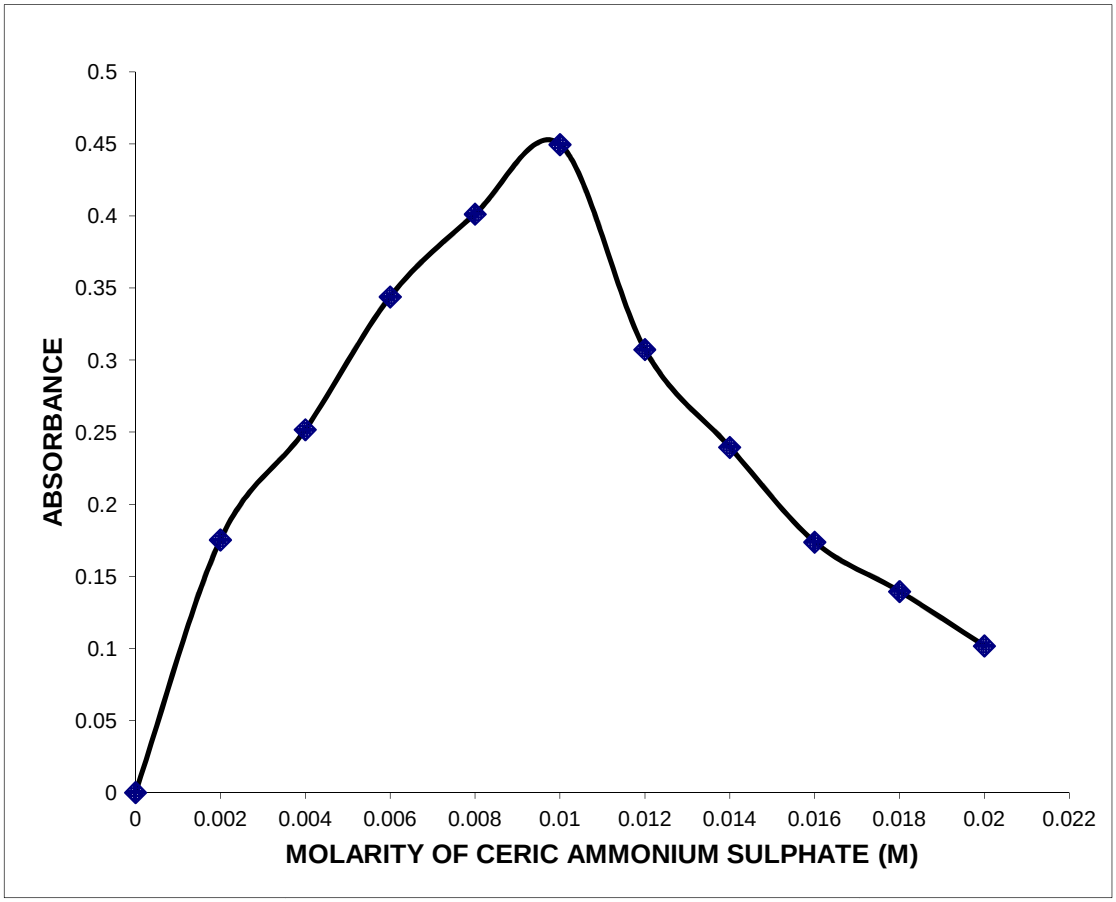


FIGURE- 8

OPTIMISATION OF CERIC AMMONIUM SULPHATE WITH DIFFERENT VOLUMES

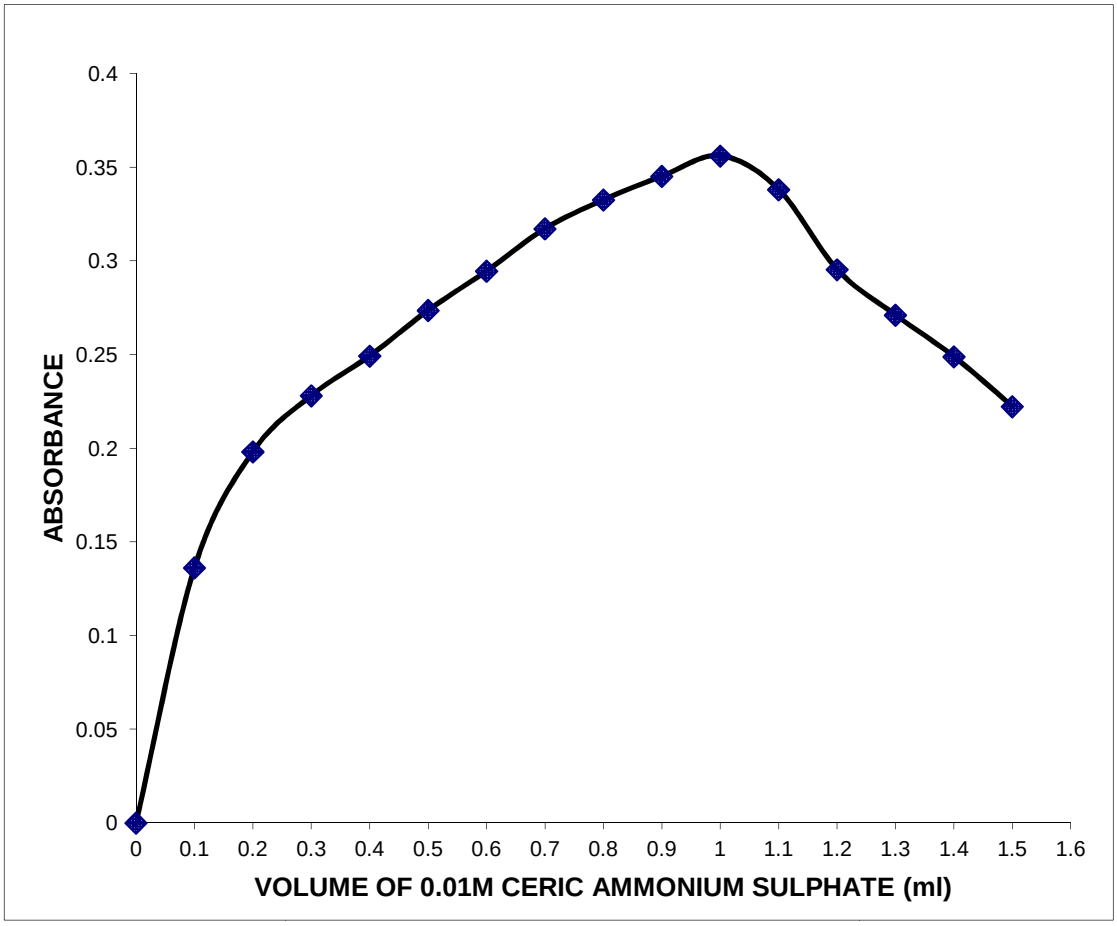


FIGURE - 9

OPTIMISATION OF MBTH REAGENT WITH DIFFERENT CONCENTRATIONS

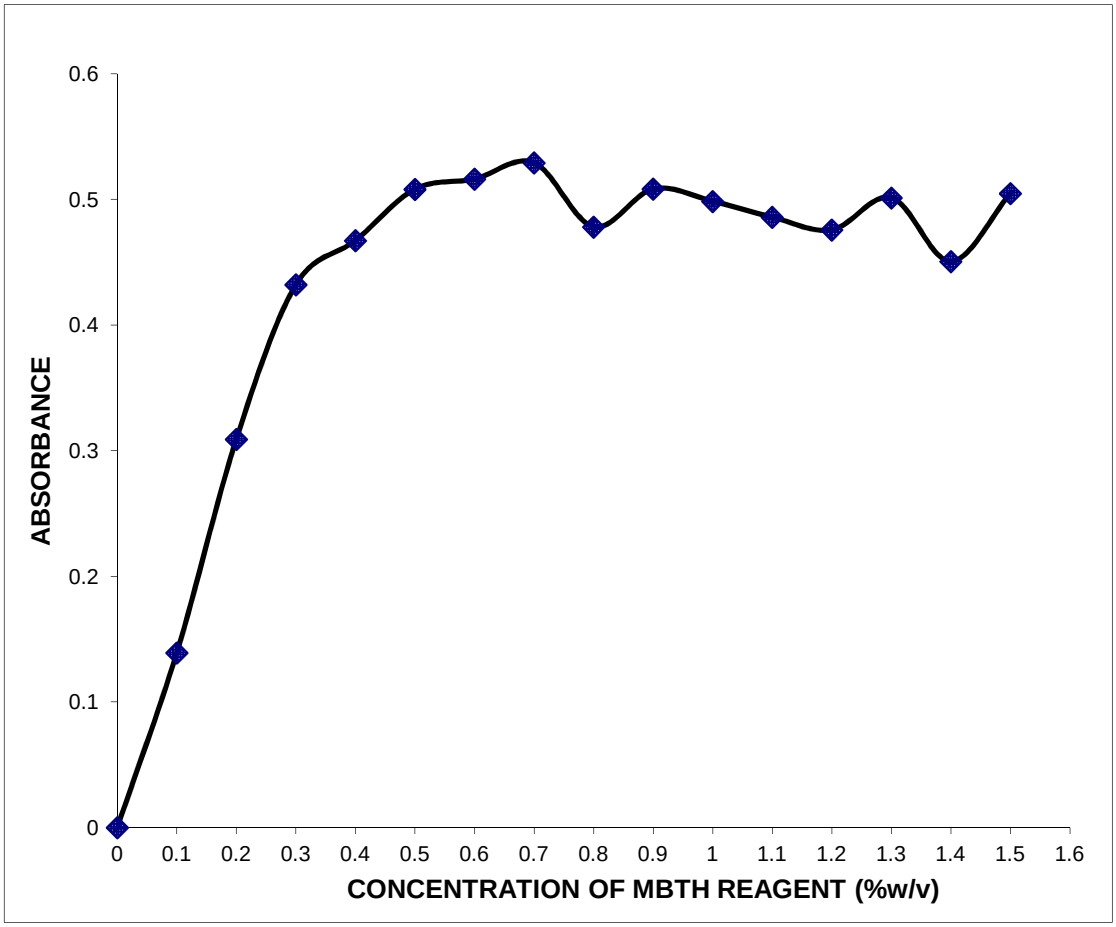


FIGURE - 10

OPTIMISATION OF MBTH REAGENT WITH DIFFERENT VOLUMES

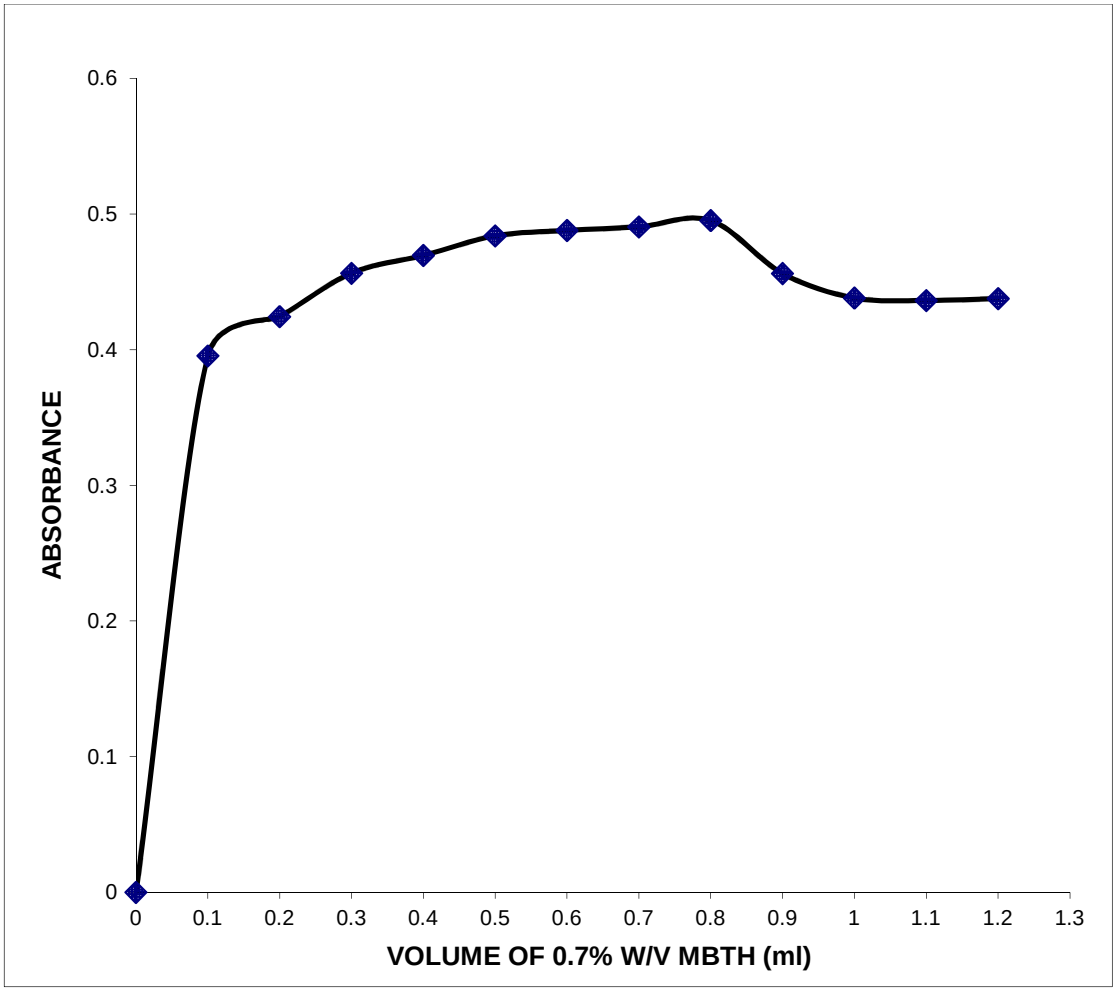


FIGURE – 11

CALIBRATION CURVE OF TAPENTADOL HYDRO CHLORIDE BY COLORIMETRIC METHOD – I

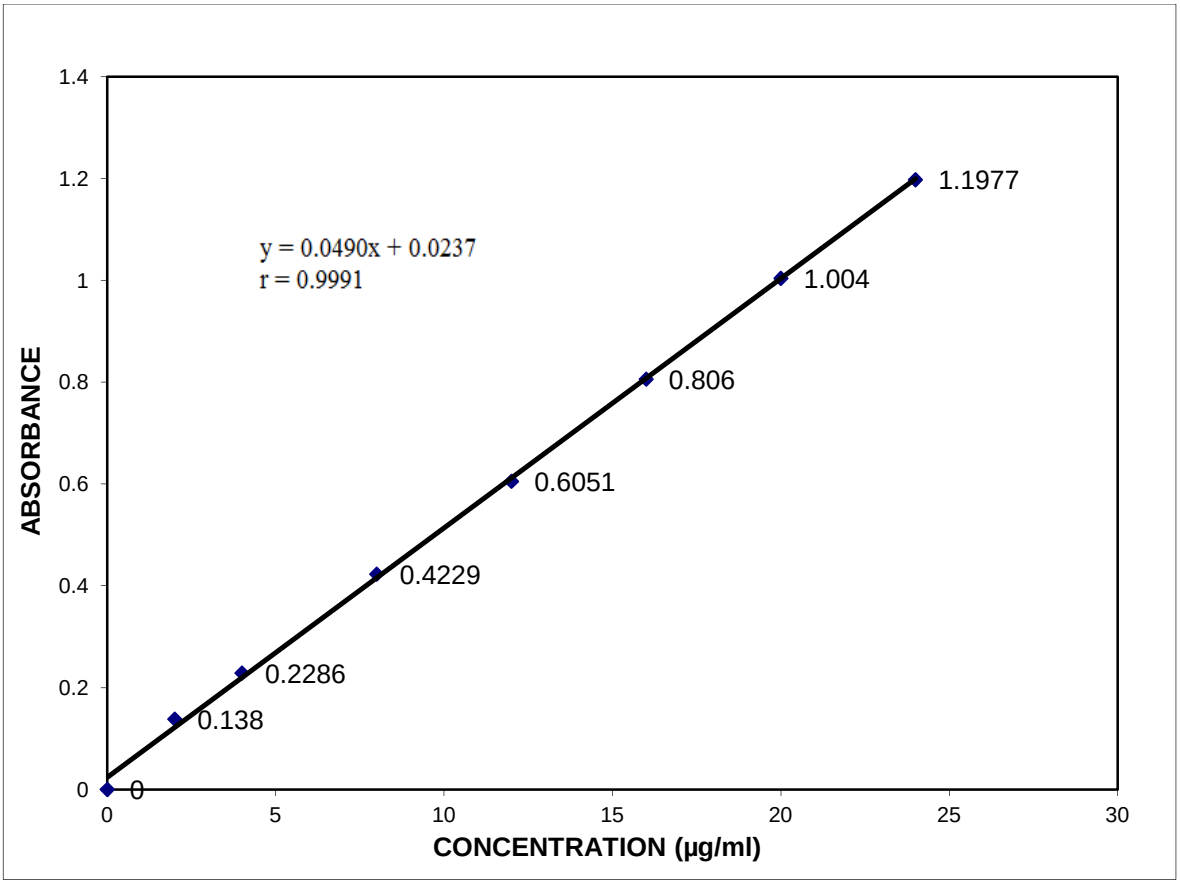


FIGURE – 12

VISIBLE SPECTRUM OF TAPENTADOL HYDROCHLORIDE BY COLORIMETRIC METHOD-II

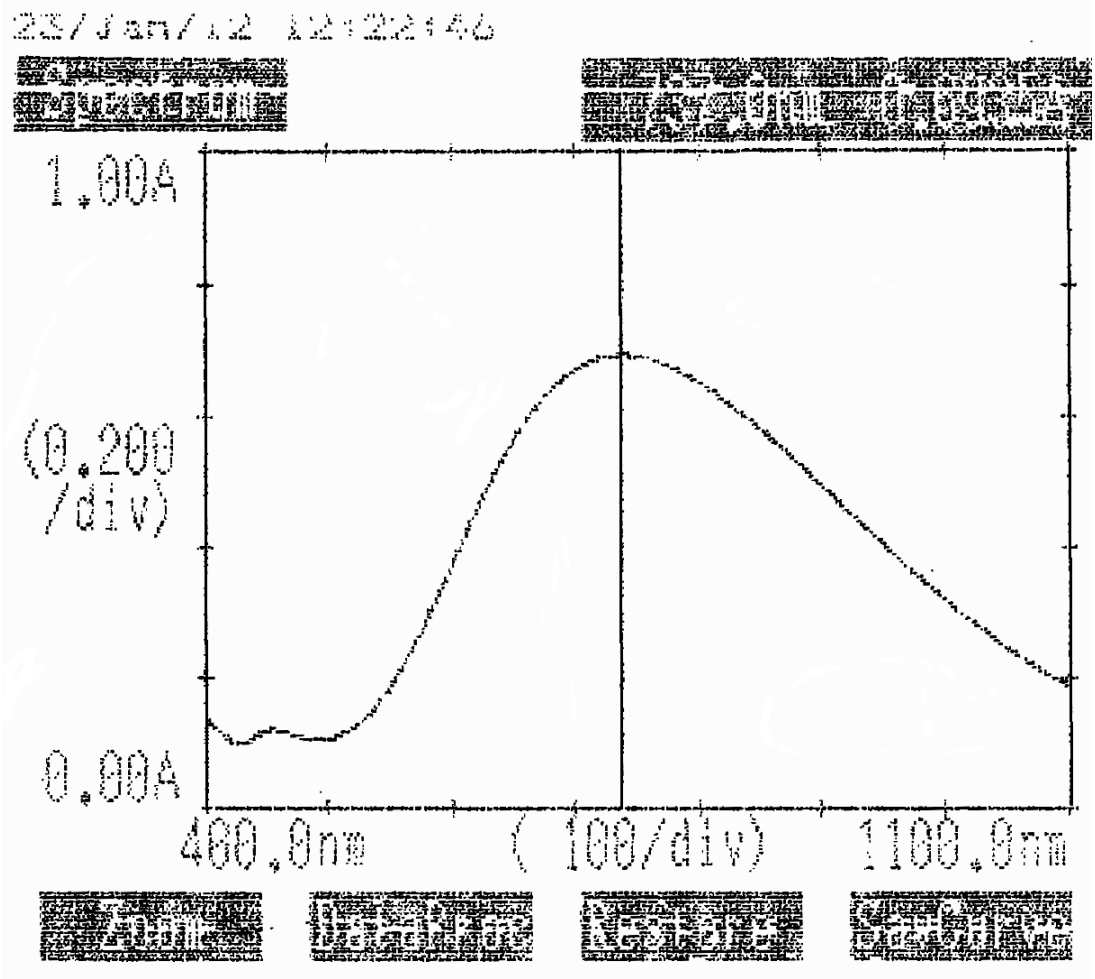


FIGURE - 13

OPTIMISATION OF FERRIC CHLORIDE WITH DIFFERENT CONCENTRATIONS

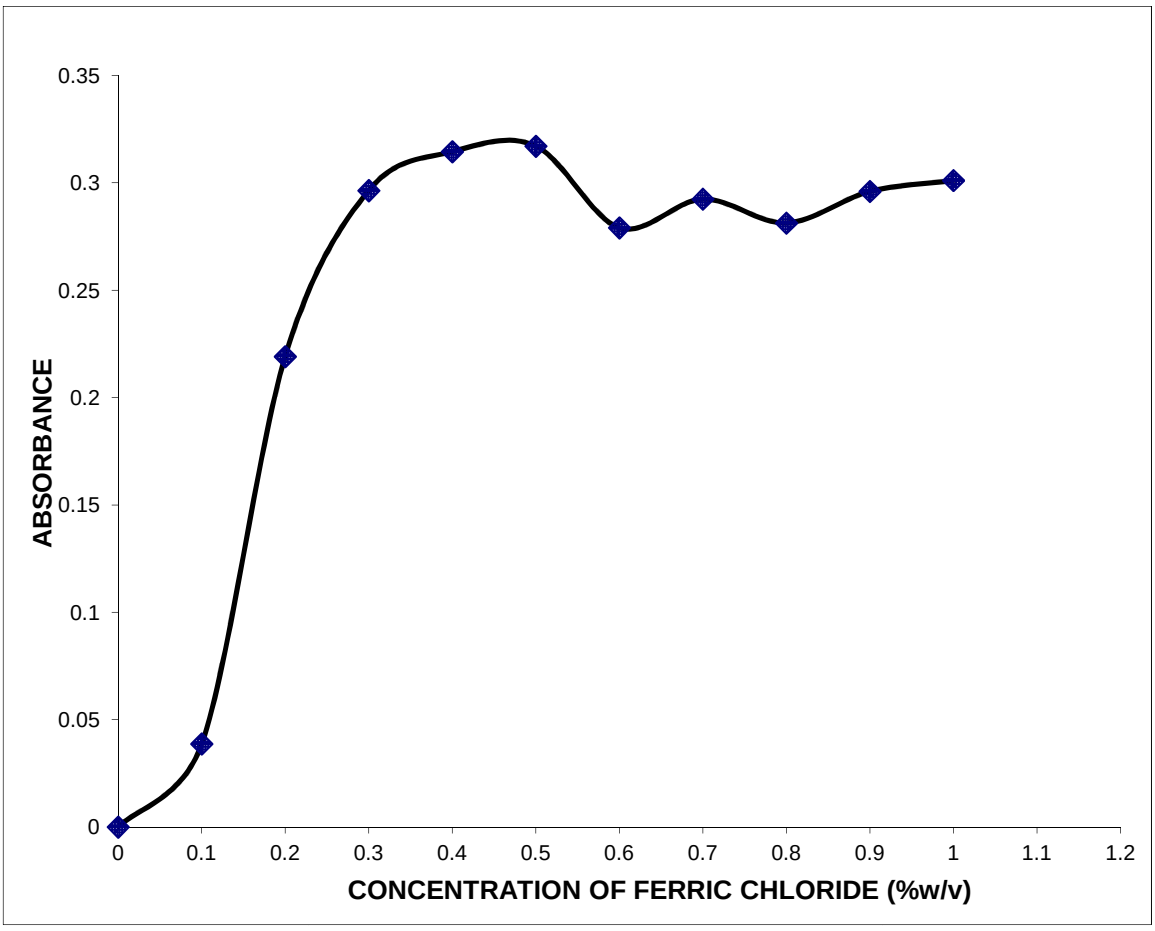


FIGURE – 14

OPTIMISATION OF FERRIC CHLORIDE WITH DIFFERENT VOLUMES

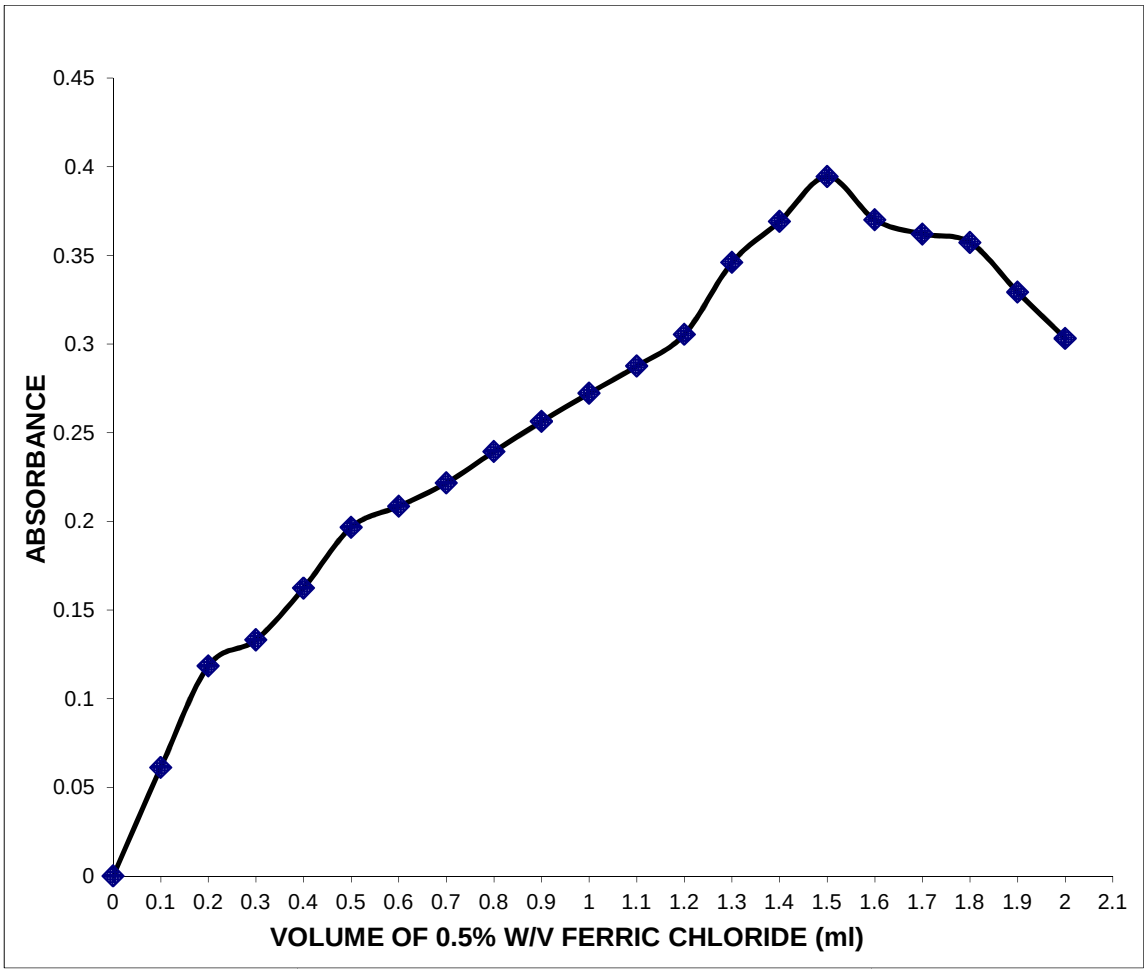


FIGURE – 15

OPTIMISATION OF POTASSIUM FERRICYANIDE WITH DIFFERENT CONCENTRATIONS

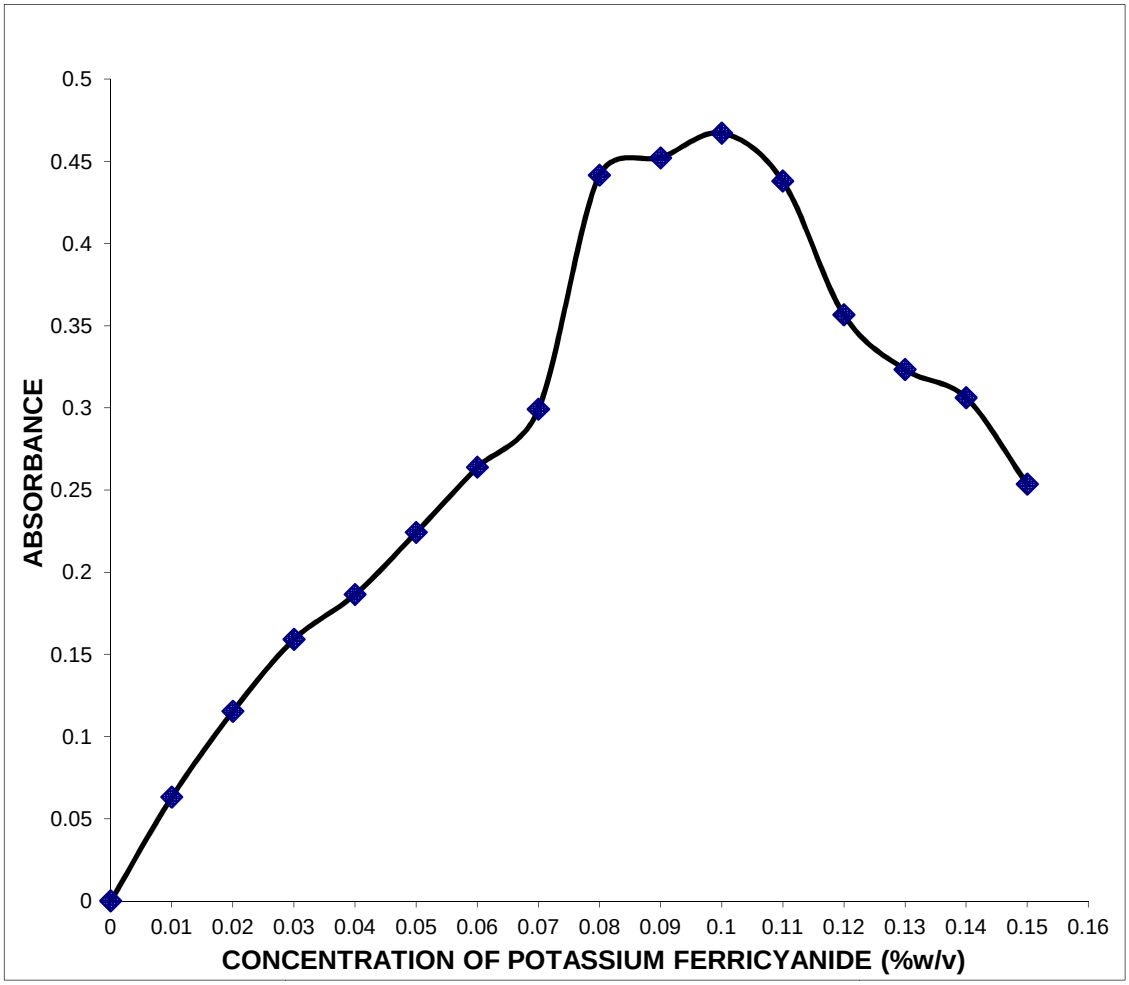


FIGURE – 16

OPTIMISATION OF POTASSIUM FERRICYANIDE WITH DIFFERENT VOLUMES

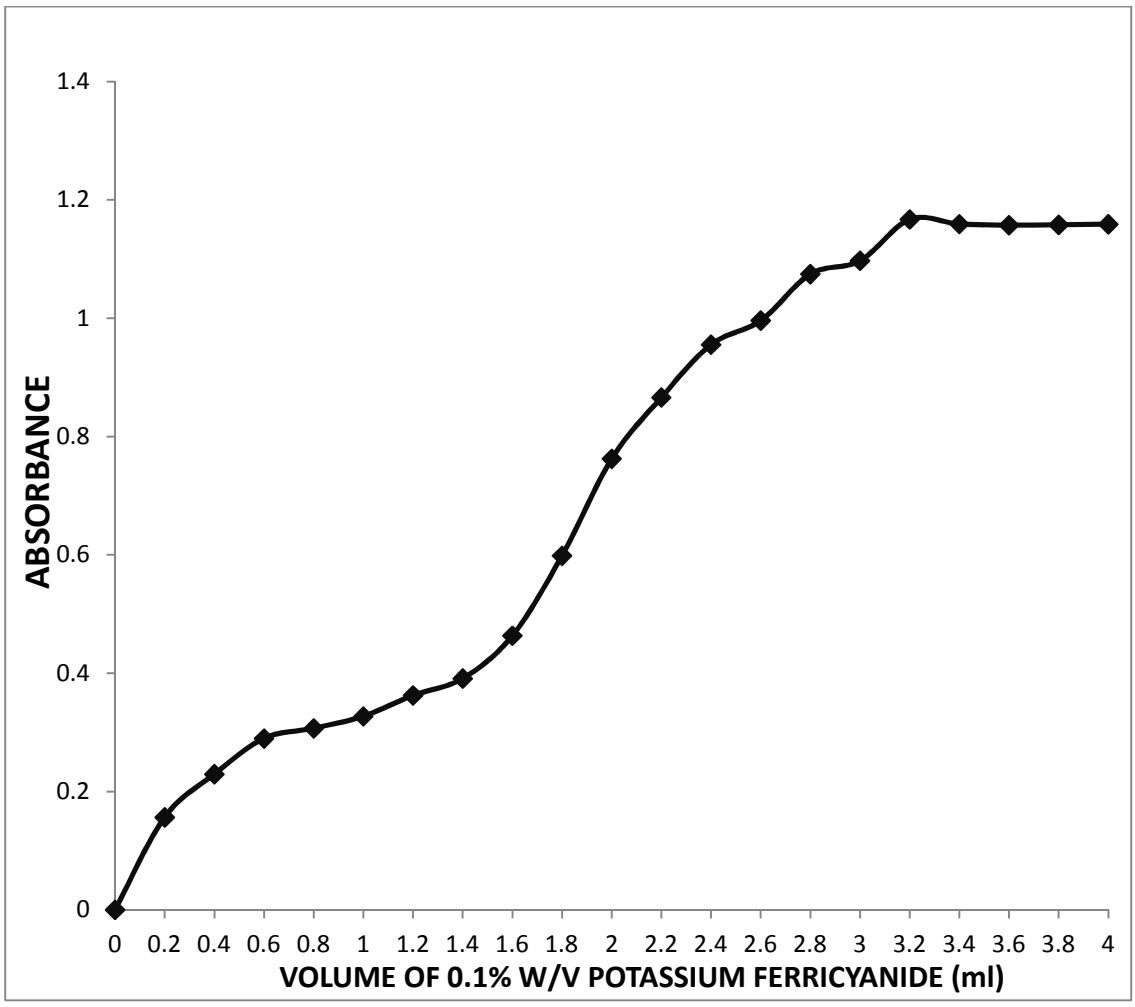


FIGURE -17

OPTIMISATION OF HYDROCHLORIC ACID WITH DIFFERENT MOLAR SOLUTIONS

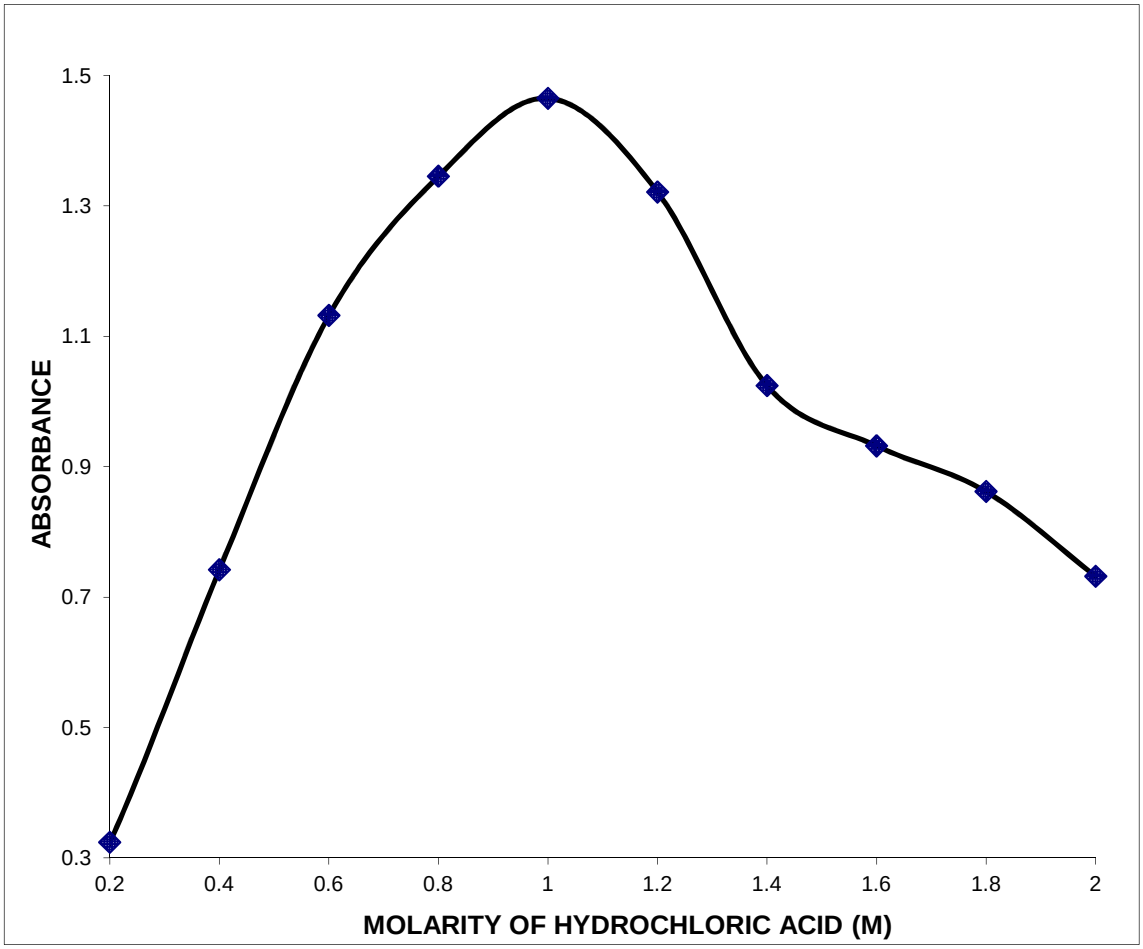


FIGURE – 18

OPTIMISATION OF HYDROCHLORIC ACID WITH DIFFERENT VOLUMES

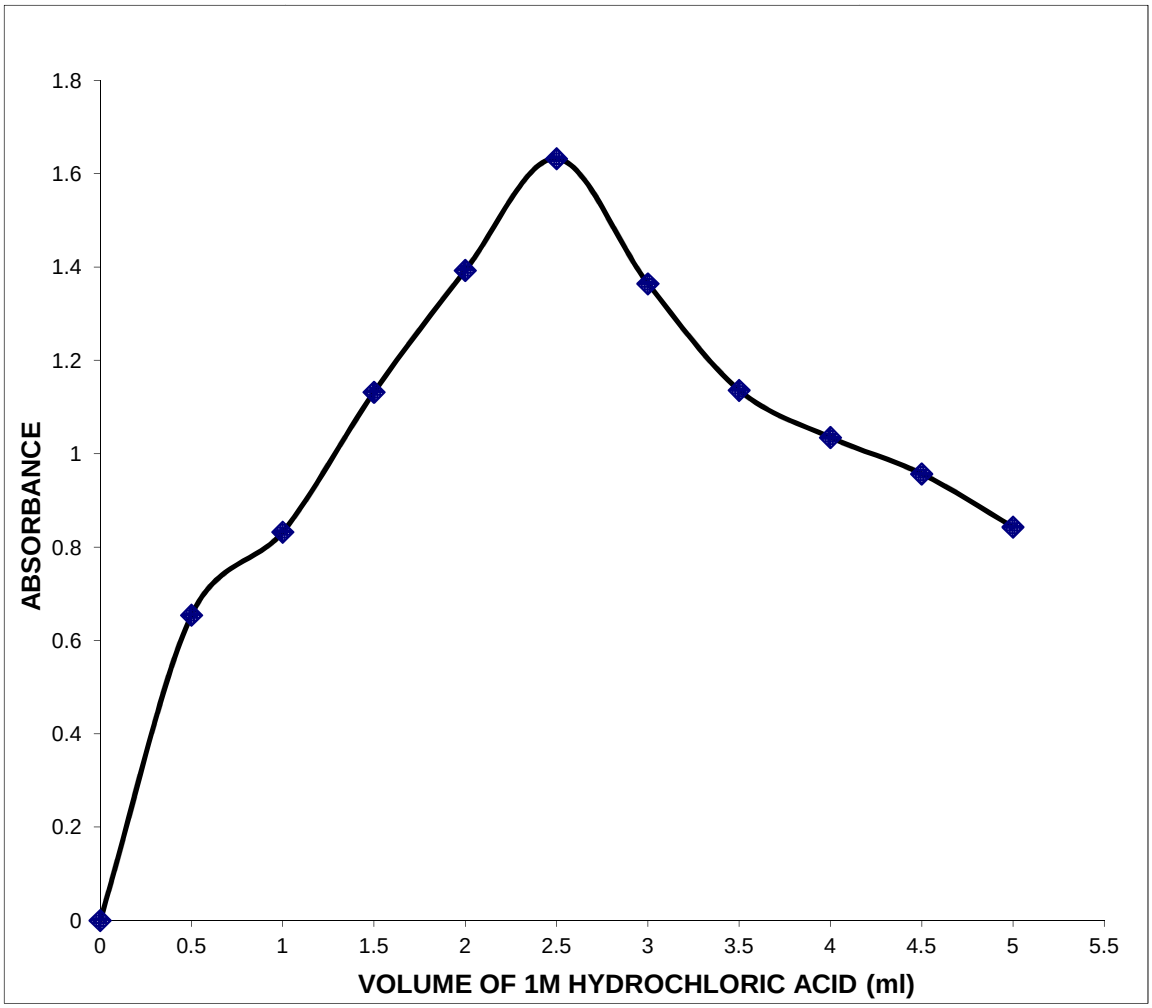


FIGURE – 19

CALIBRATION CURVE OF TAPENTADOL HYDROCHLORIDE BY COLORIMETRIC METHOD-II

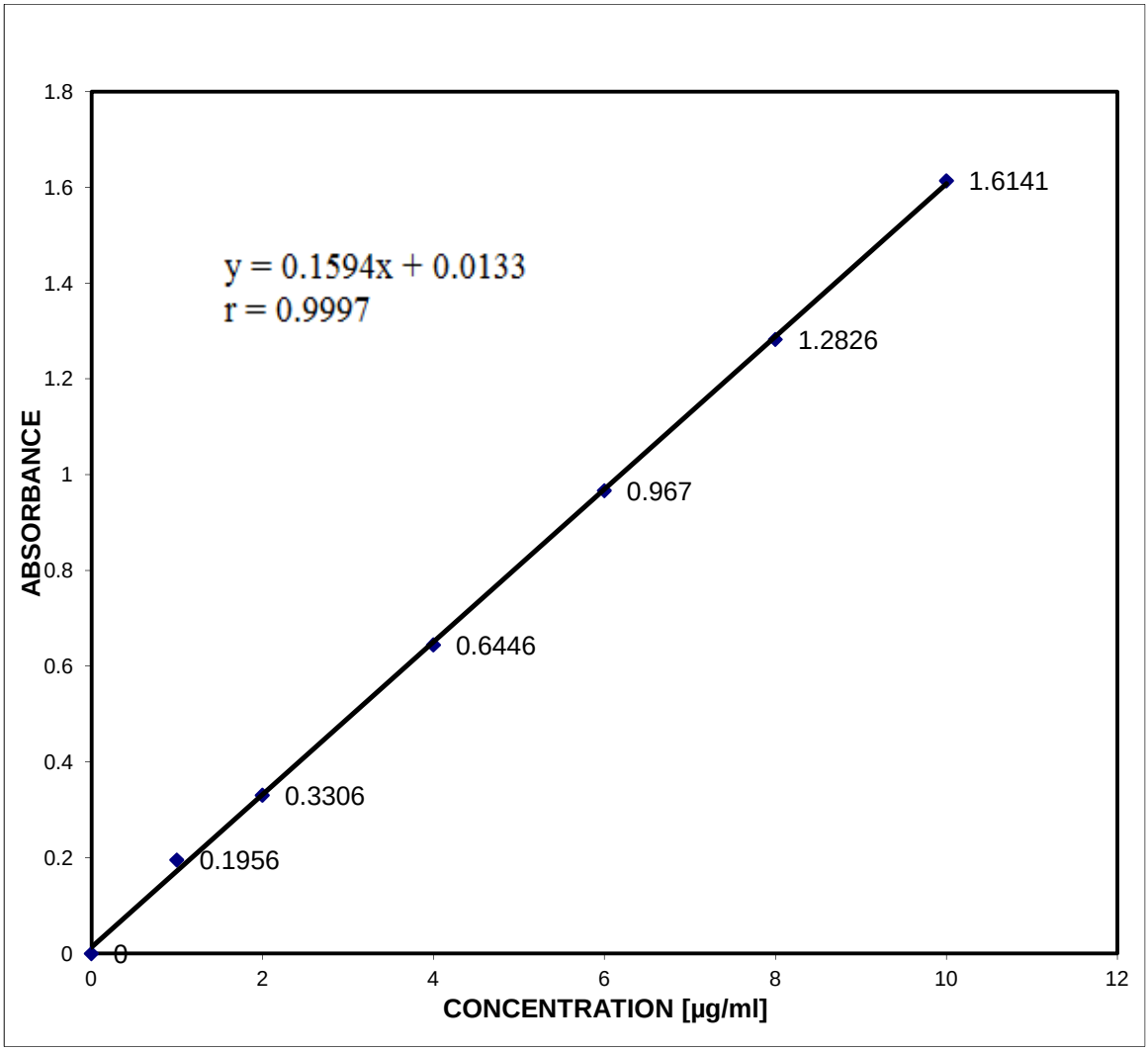


FIGURE -20

SPECTRAL CONFORMATION OF STANDARD TAPENTADOL HYDROCHLORIDE WITH FORMULATION BY HPTLC

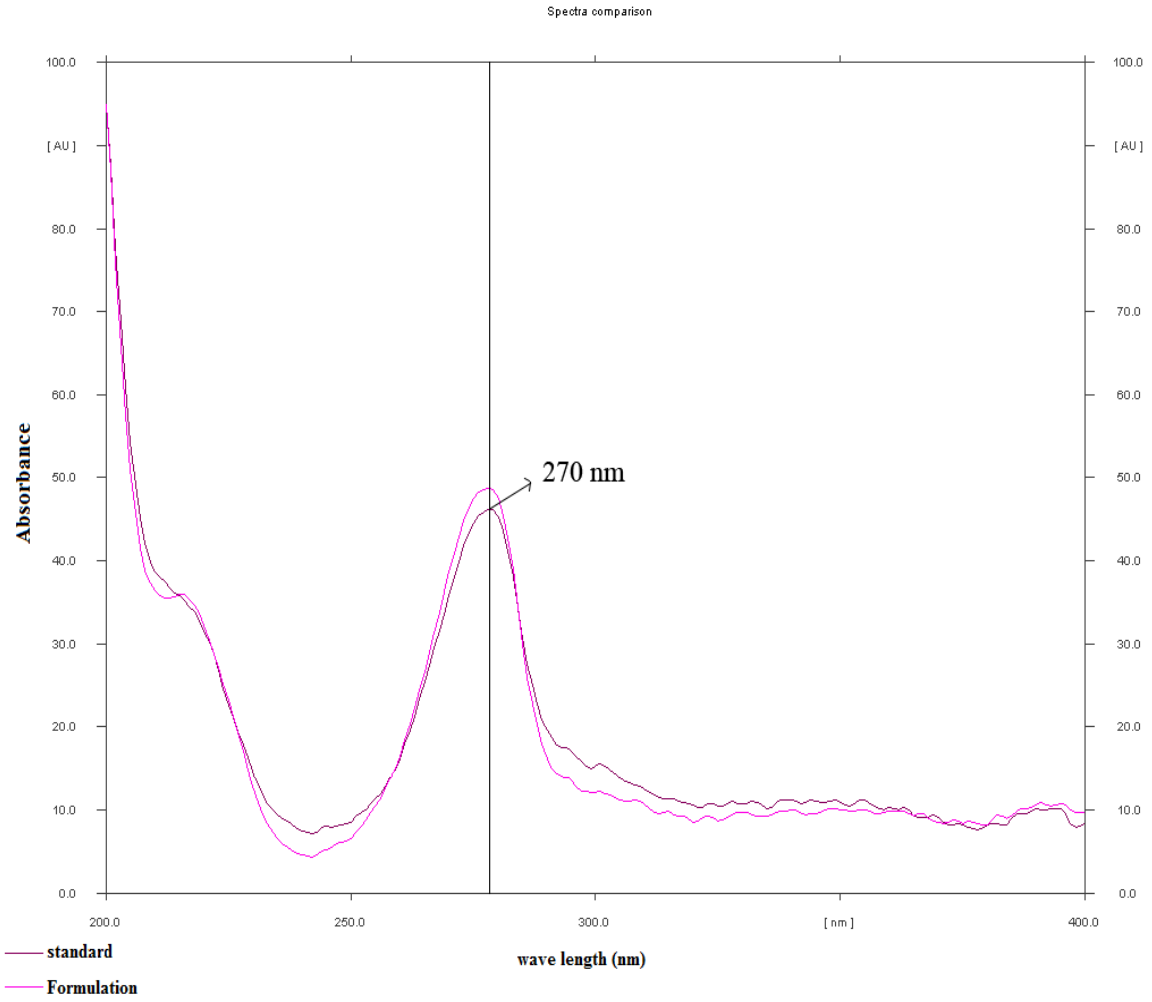
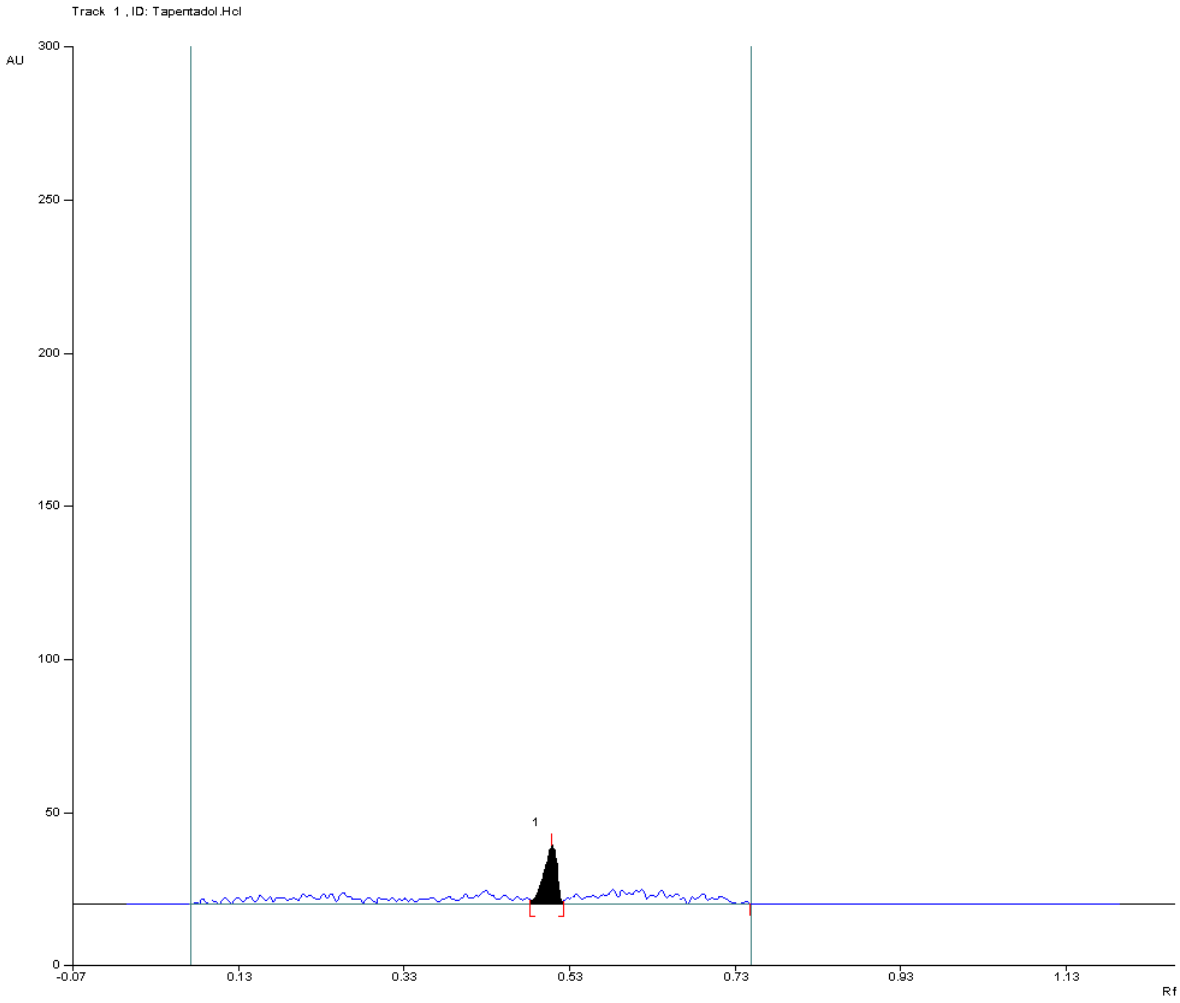


FIGURE - 21

LINEARITY CHROMATOGRAM FOR TAPENTADOL HYDROCHLORIDE BY HPTLC

(100 ng/ µl**)**

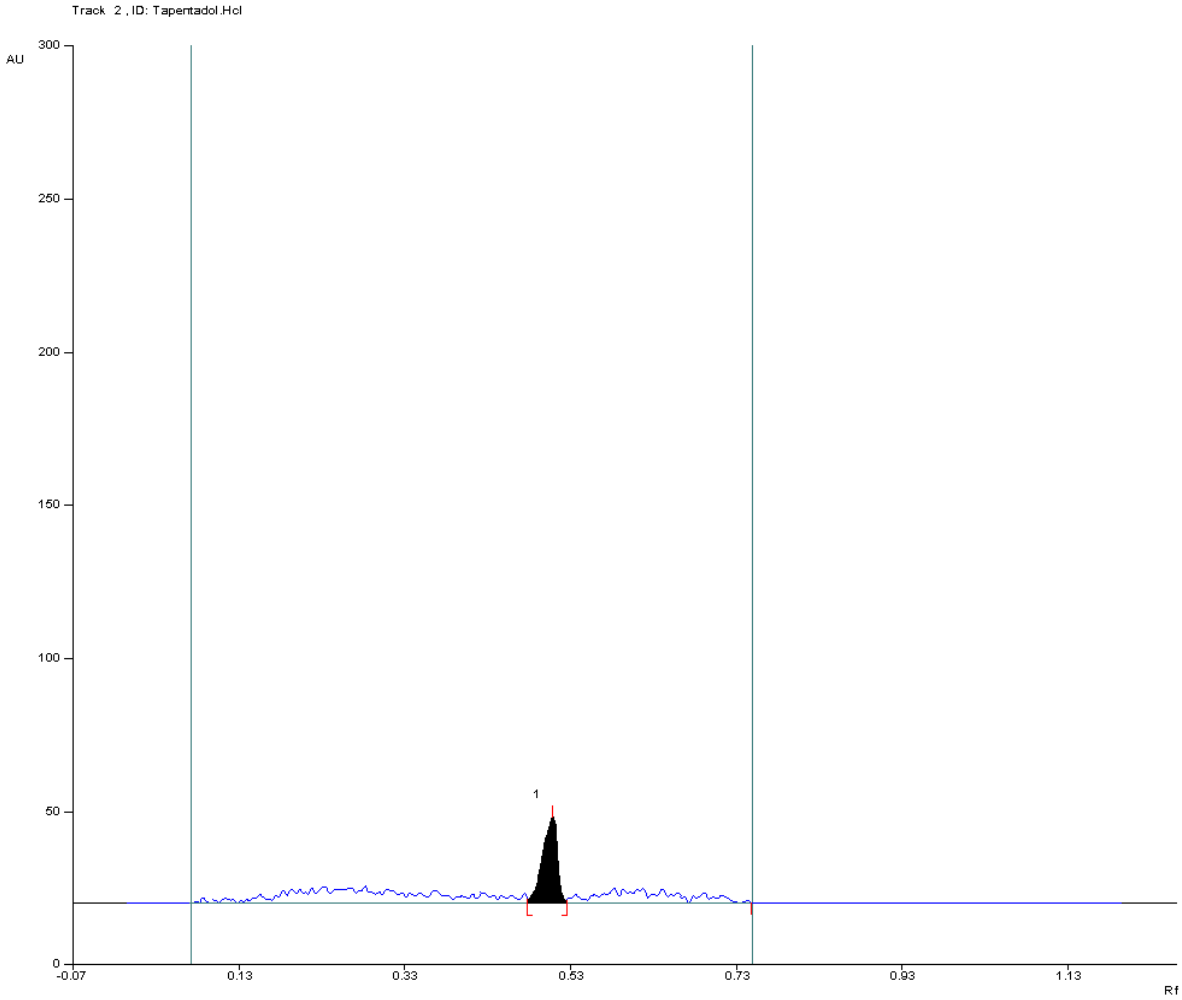


Peak	Start Rf	Start Height	Max Rf	Max Height	Max%	End Rf	End Height	Area	Area %
1	0.46	2.0	0.50	22.8	100%	0.52	1.5	207.2	100%

FIGURE - 22

LINEARITY CHROMATOGRAM FOR TAPENTADOL HYDROCHLORIDE BY HPTLC

(200 ng/ µl**)**

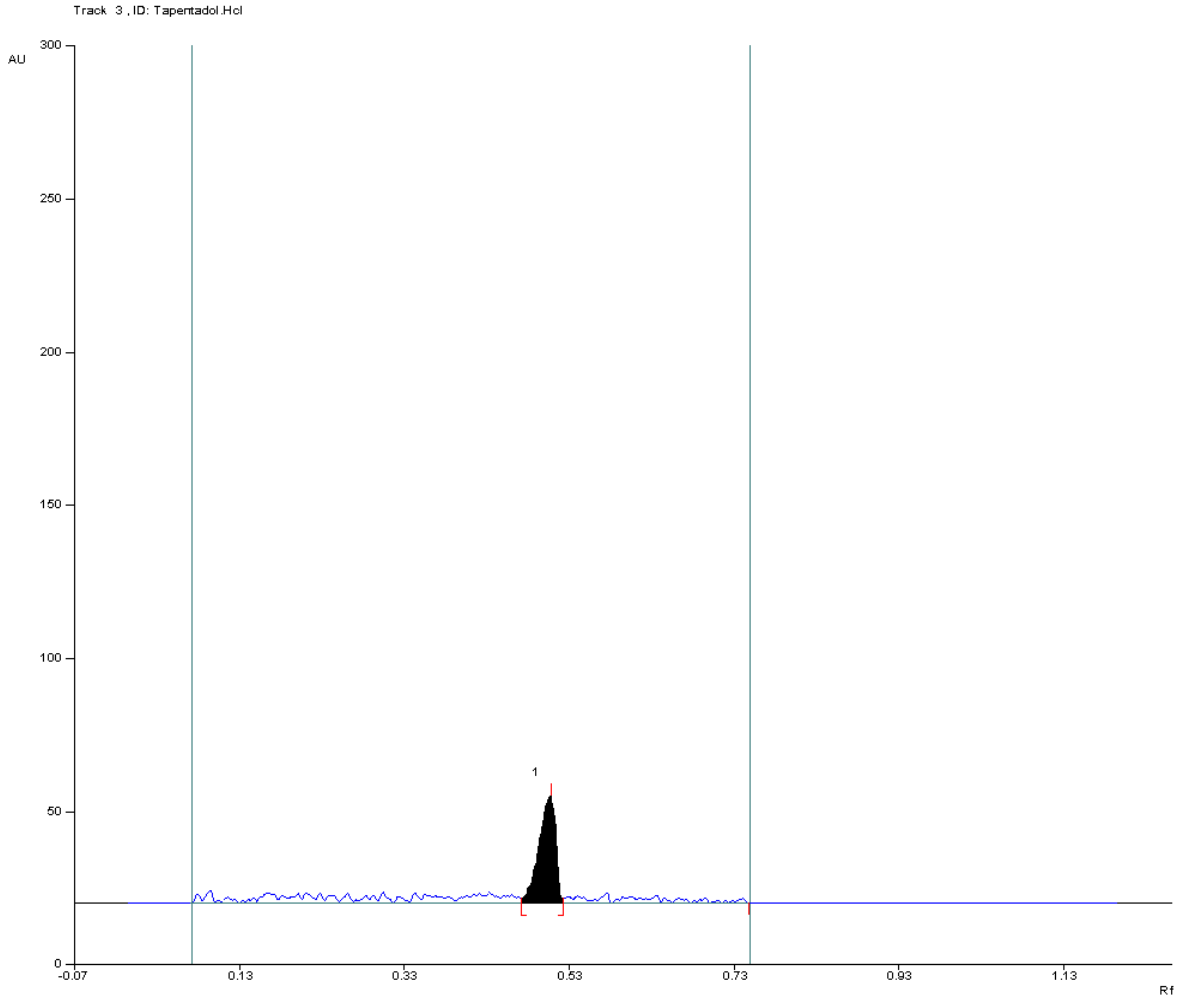


Peak	Start Rf	Start Height	Max Rf	Max Height	Max%	End Rf	End Height	Area	Area %
1	0.46	1.6	0.51	29.2	100%	0.53	1.8	382.1	100%

FIGURE - 23

LINEARITY CHROMATOGRAM FOR TAPENTADOL HYDROCHLORIDE BY HPTLC

(300 ng/ µl**)**

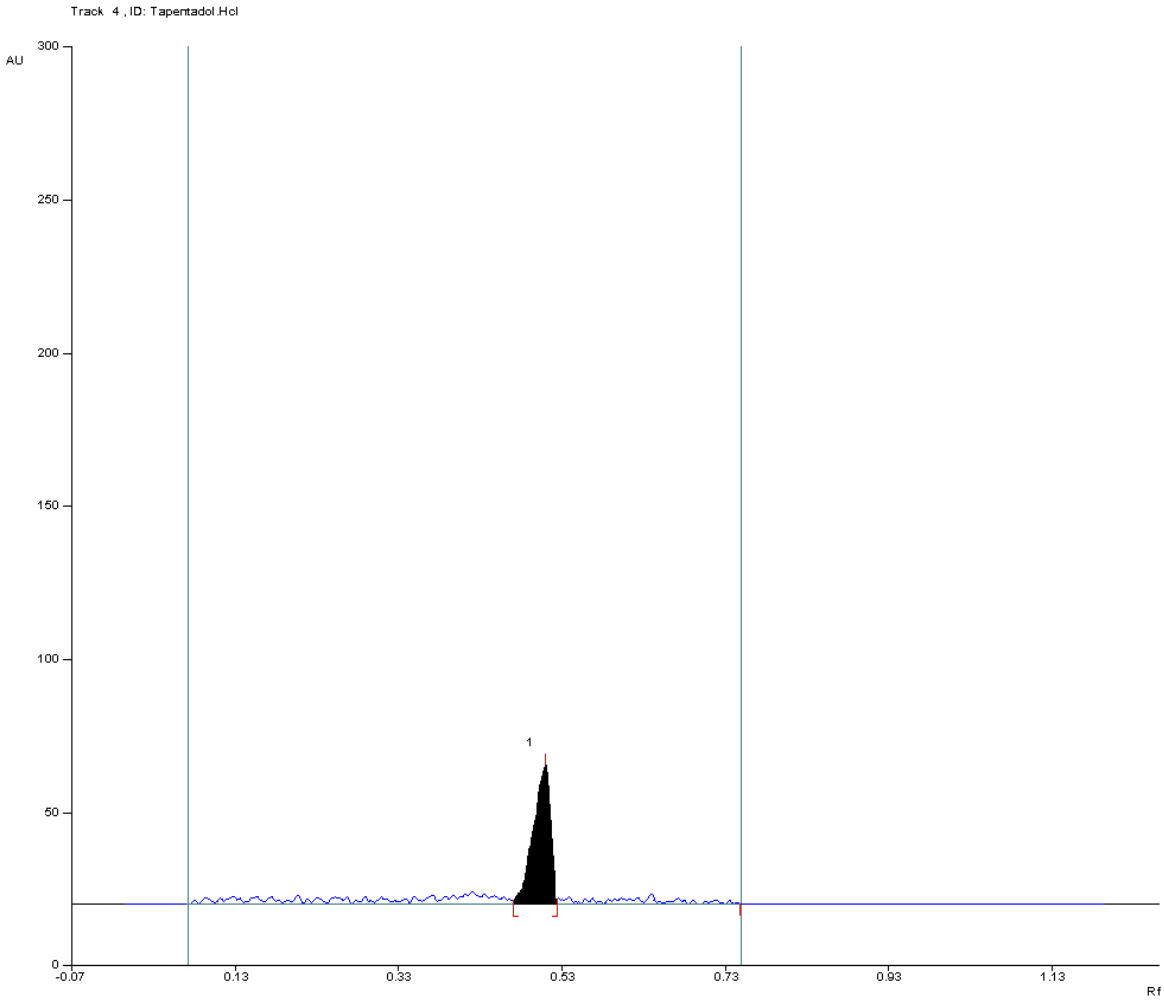


Peak	Start Rf	Start Height	Max Rf	Max Height	Max%	End Rf	End Height	Area	Area %
1	0.45	2.2	0.50	37.7	100%	0.52	1.2	554.91	100%

FIGURE - 24

LINEARITY CHROMATOGRAM FOR TAPENTADOL HYDROCHLORIDE BY HPTLC

(400 ng/ µl)

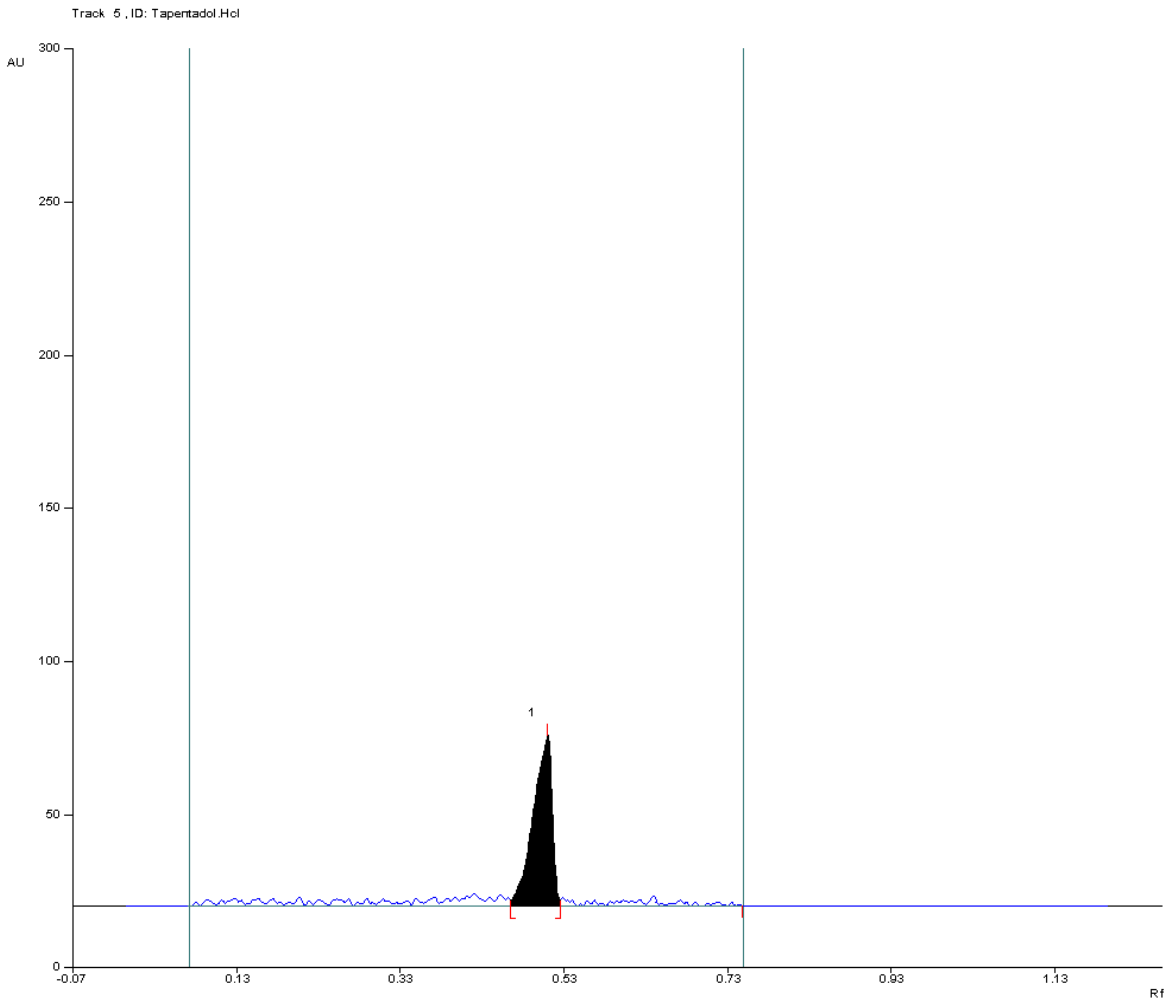


Peak	Start Rf	Start Height	Max Rf	Max Height	Max%	End Rf	End Height	Area	Area %
1	0.46	1.6	0.51	46.2	100%	0.53	2.1	728.66	100%

FIGURE - 25

LINEARITY CHROMATOGRAM FOR TAPENTADOL HYDROCHLORIDE BY HPTLC

(500 ng/ µl)

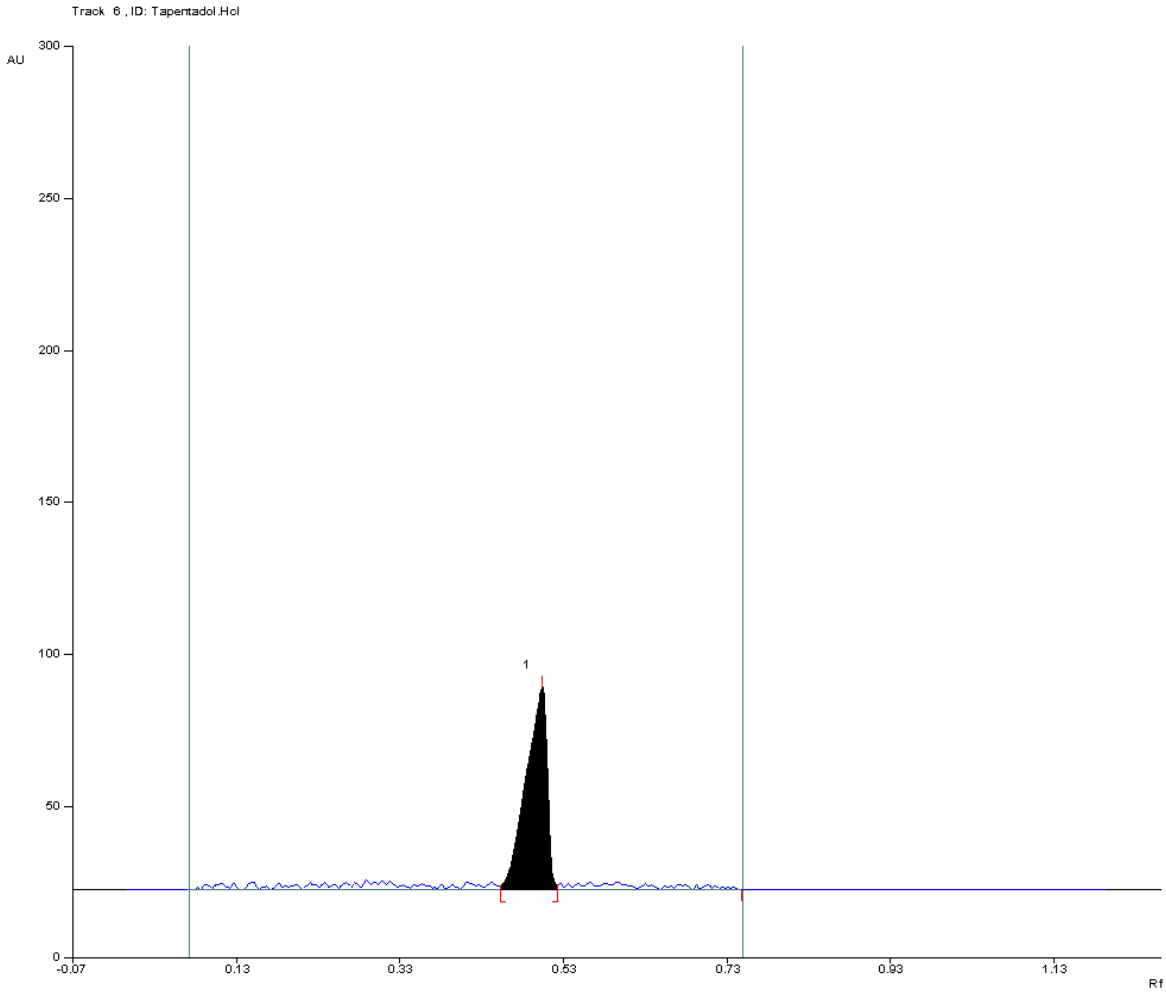


Peak	Start Rf	Start Height	Max Rf	Max Height	Max%	End Rf	End Height	Area	Area %
1	0.45	2.8	0.49	56.8	100%	0.53	2.3	893.98	100%

FIGURE - 26

LINEARITY CHROMATOGRAM FOR TAPENTADOL HYDROCHLORIDE BY HPTLC

(600 ng/ µl)



Peak	Start Rf	Start Height	Max Rf	Max Height	Max%	End Rf	End Height	Area	Area %
1	0.45	2.7	0.49	67.1	100%	0.54	3.1	1062.1	100%

FIGURE - 27

CALIBRATION CURVE FOR TAPENTADOL HYDROCHLORIDE BY HPTLC METHOD

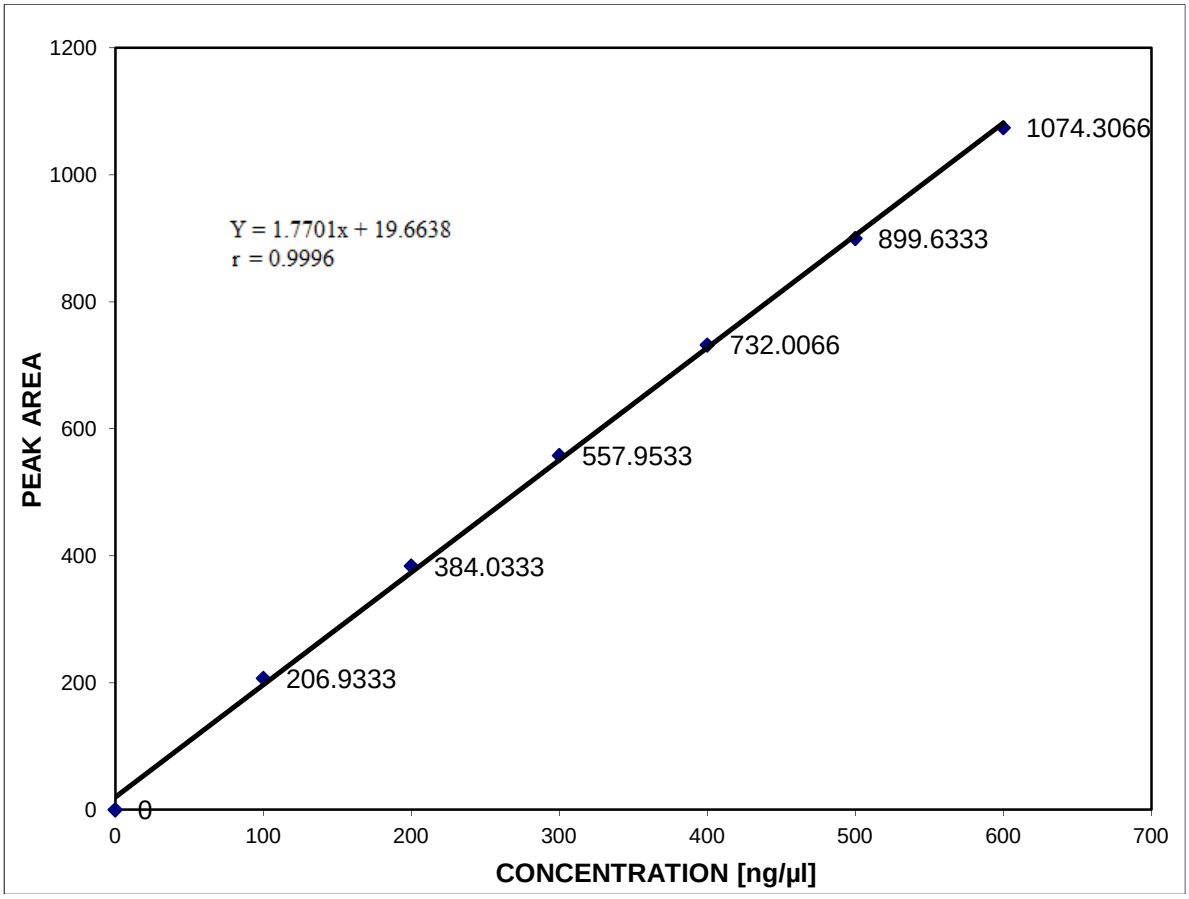
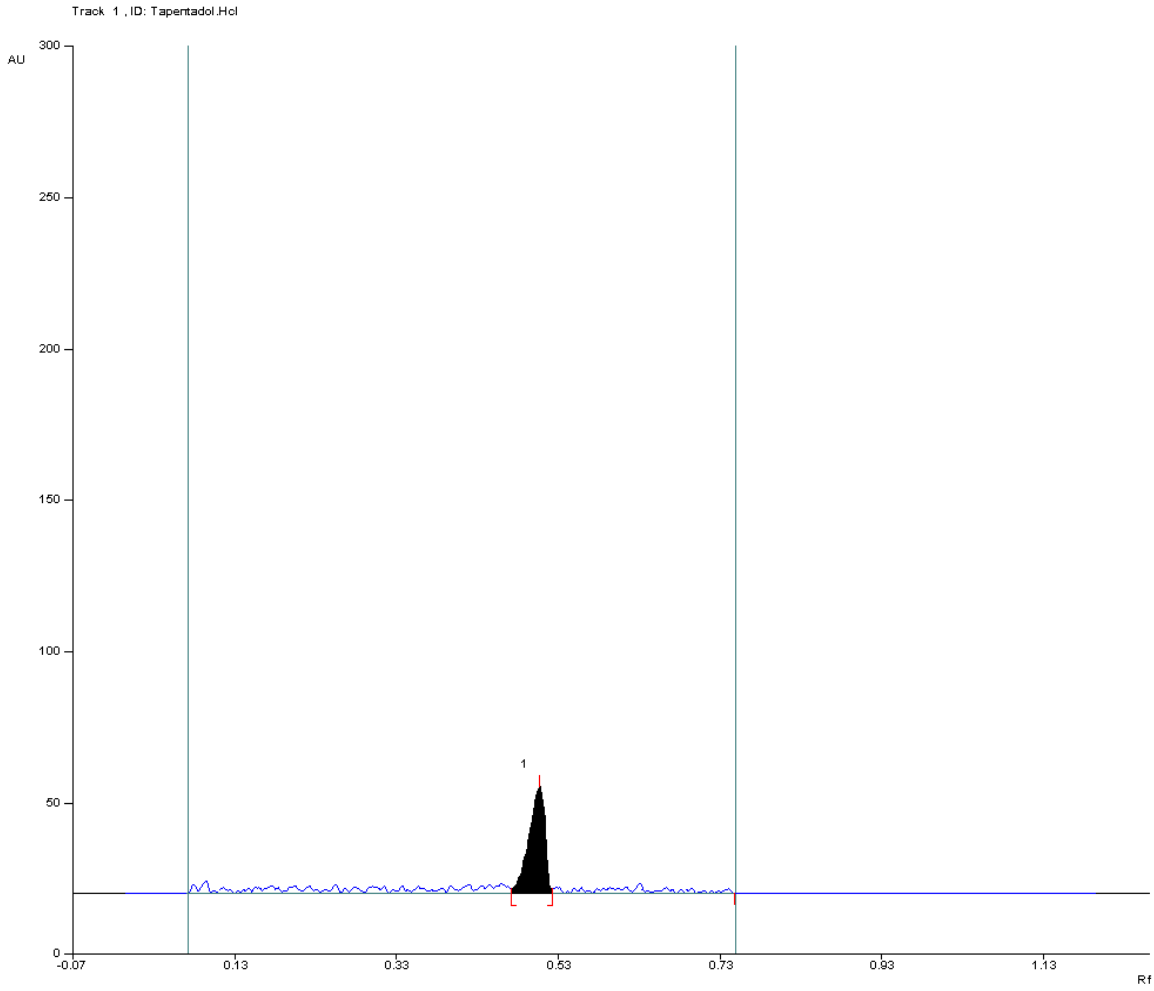


FIGURE – 28

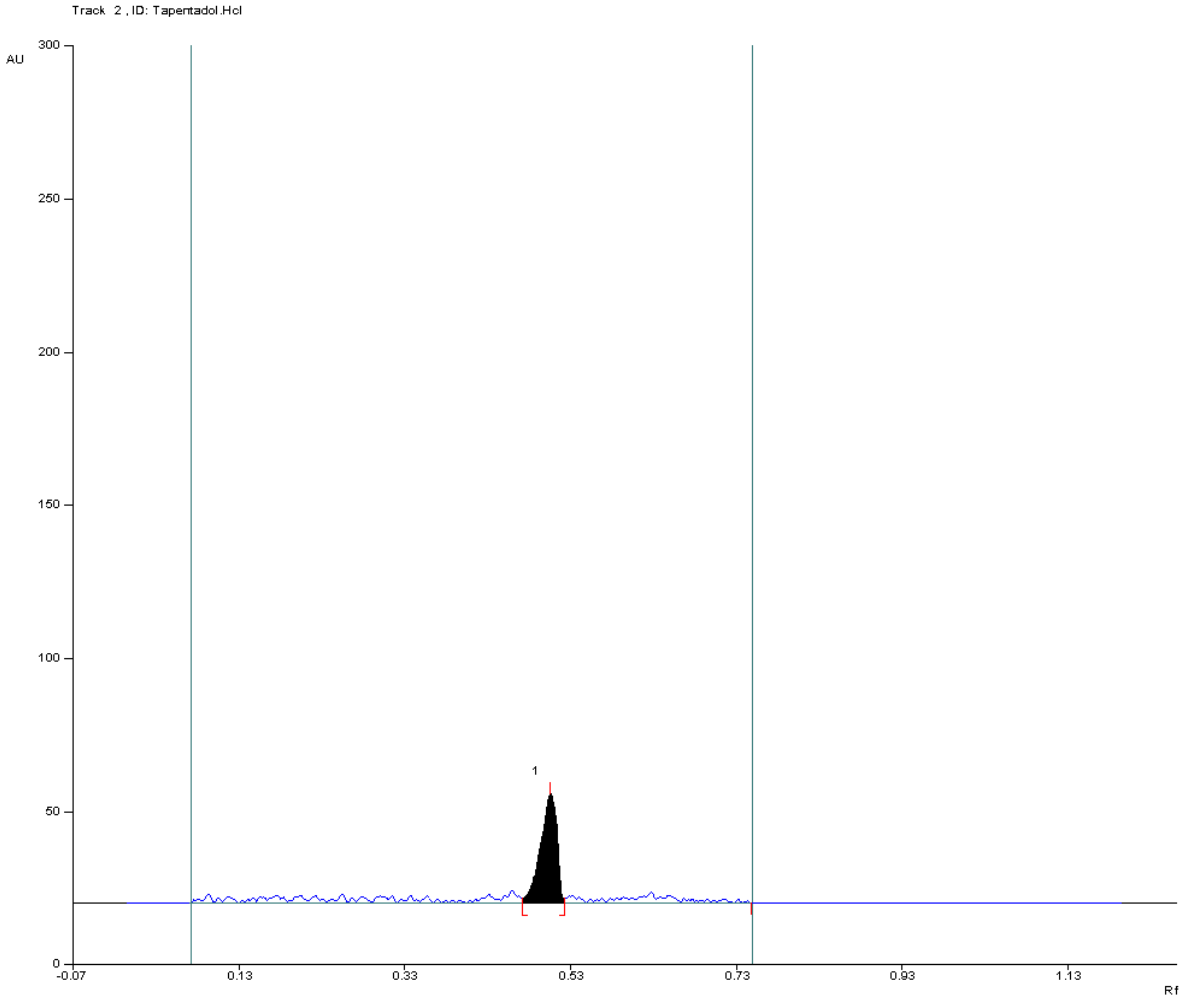
CHROMATOGRAM FOR FORMULATION ANALYSIS BY HPTLC REPEATABILITY 1



Peak	Start Rf	Start Height	Max Rf	Max Height	Max%	End Rf	End Height	Area	Area %
1	0.46	2.0	0.49	37.4	100%	0.52	1.8	552.1	100%

FIGURE – 29

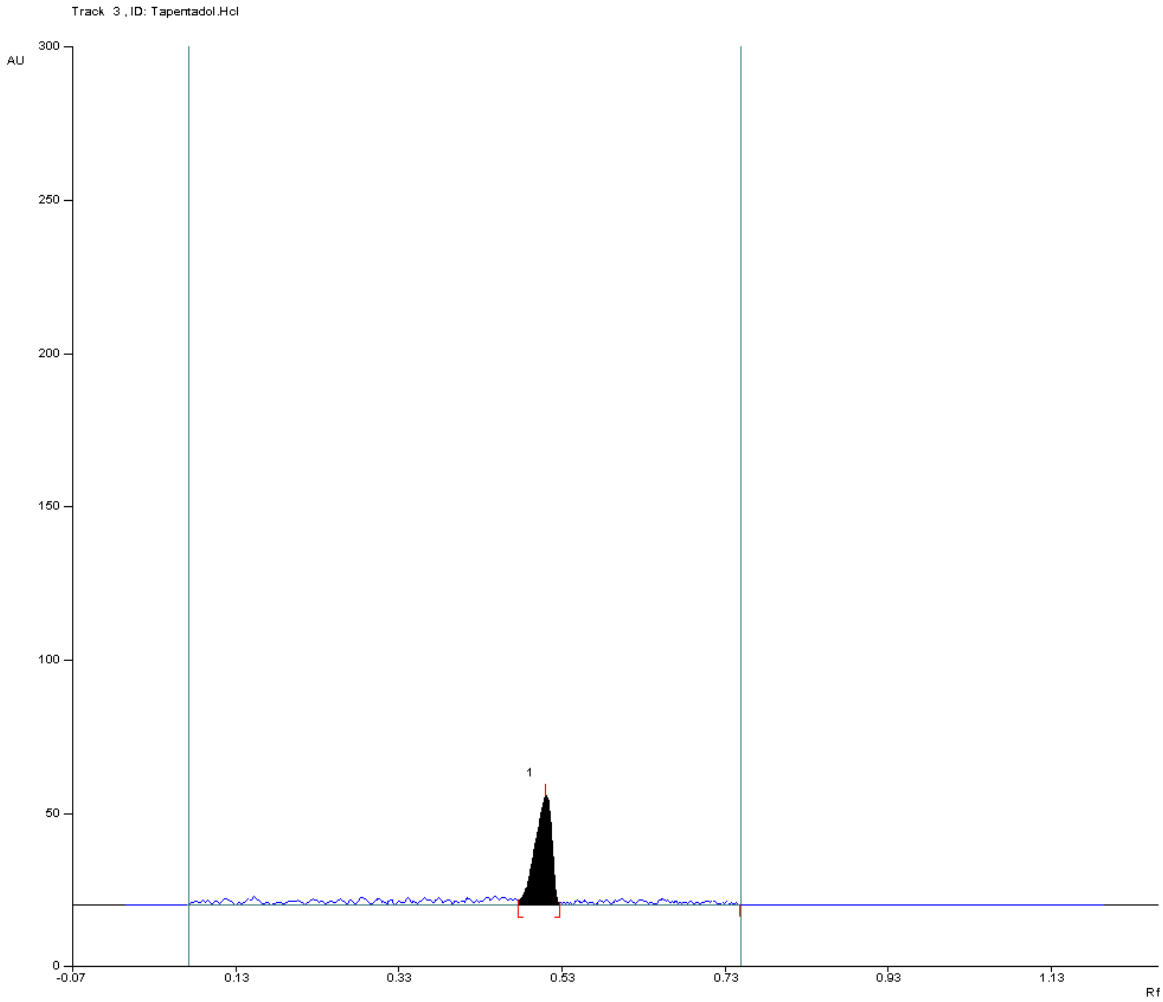
CHROMATOGRAM FOR FORMULATION ANALYSIS BY HPTLC REPEATABILITY 2



Peak	Start Rf	Start Height	Max Rf	Max Height	Max%	End Rf	End Height	Area	Area %
1	0.46	2.1	0.50	38.2	100%	0.53	1.6	551.0	100%

FIGURE – 30

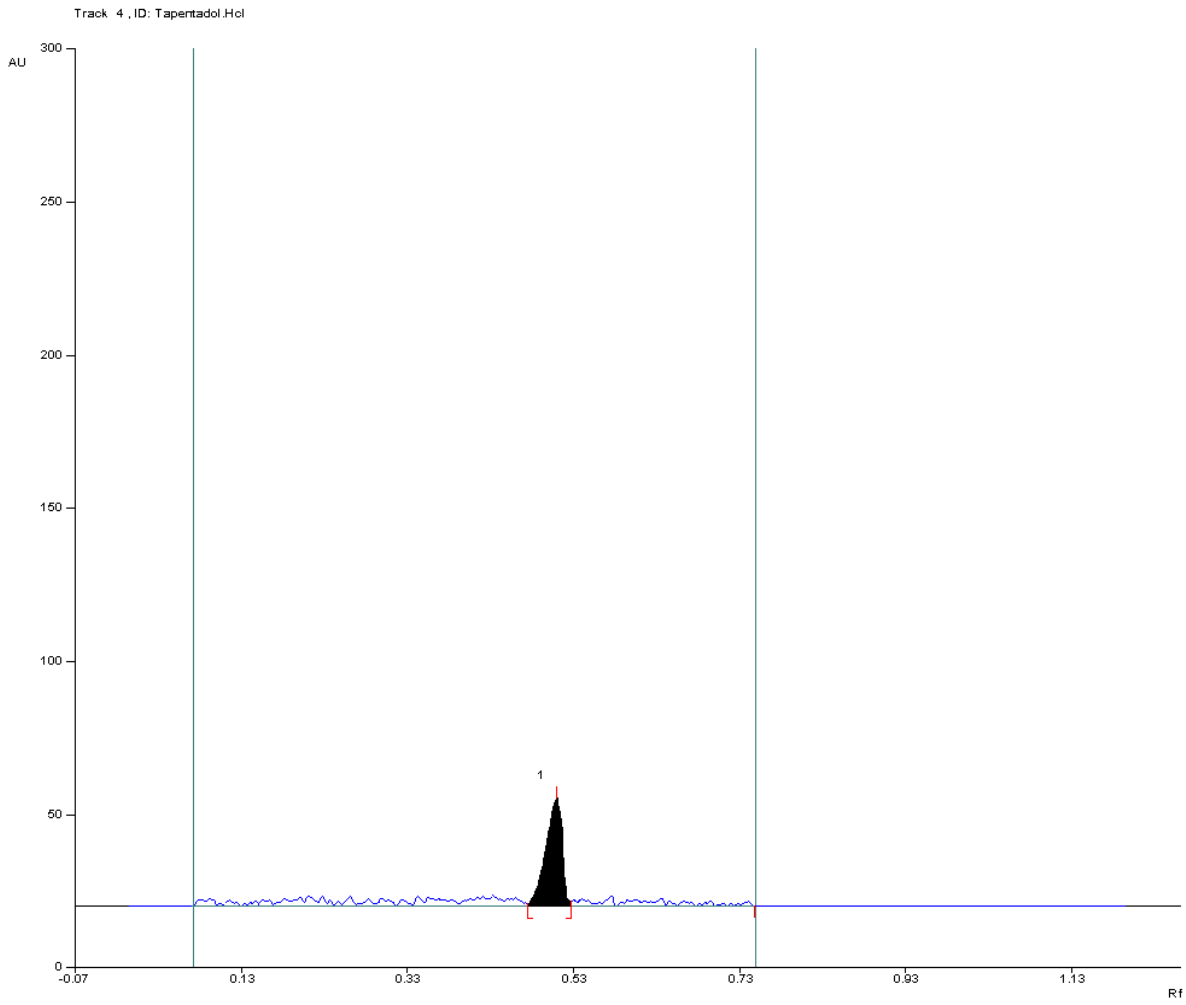
CHROMATOGRAM FOR FORMULATION ANALYSIS BY HPTLC REPEATABILITY 3



Peak	Start Rf	Start Height	Max Rf	Max Height	Max%	End Rf	End Height	Area	Area %
1	0.47	2.0	0.50	37.8	100%	0.53	0.9	548.9	100%

FIGURE - 31

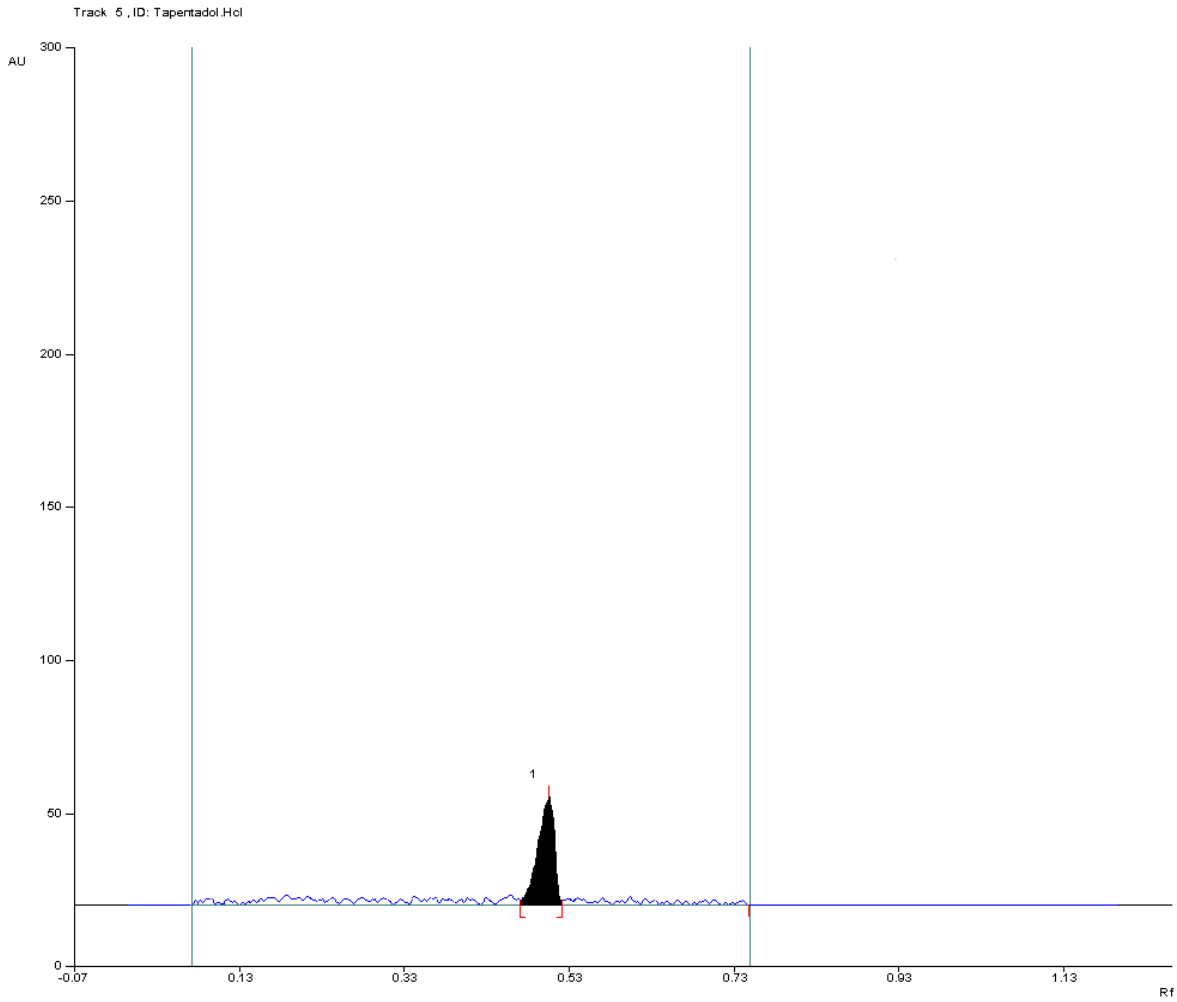
CHROMATOGRAM FOR FORMULATION ANALYSIS BY HPTLC REPEATABILITY 4



Peak	Start Rf	Start Height	Max Rf	Max Height	Max%	End Rf	End Height	Area	Area %
1	0.47	0.8	0.51	36.9	100%	0.54	2.2	550.7	100%

FIGURE – 32

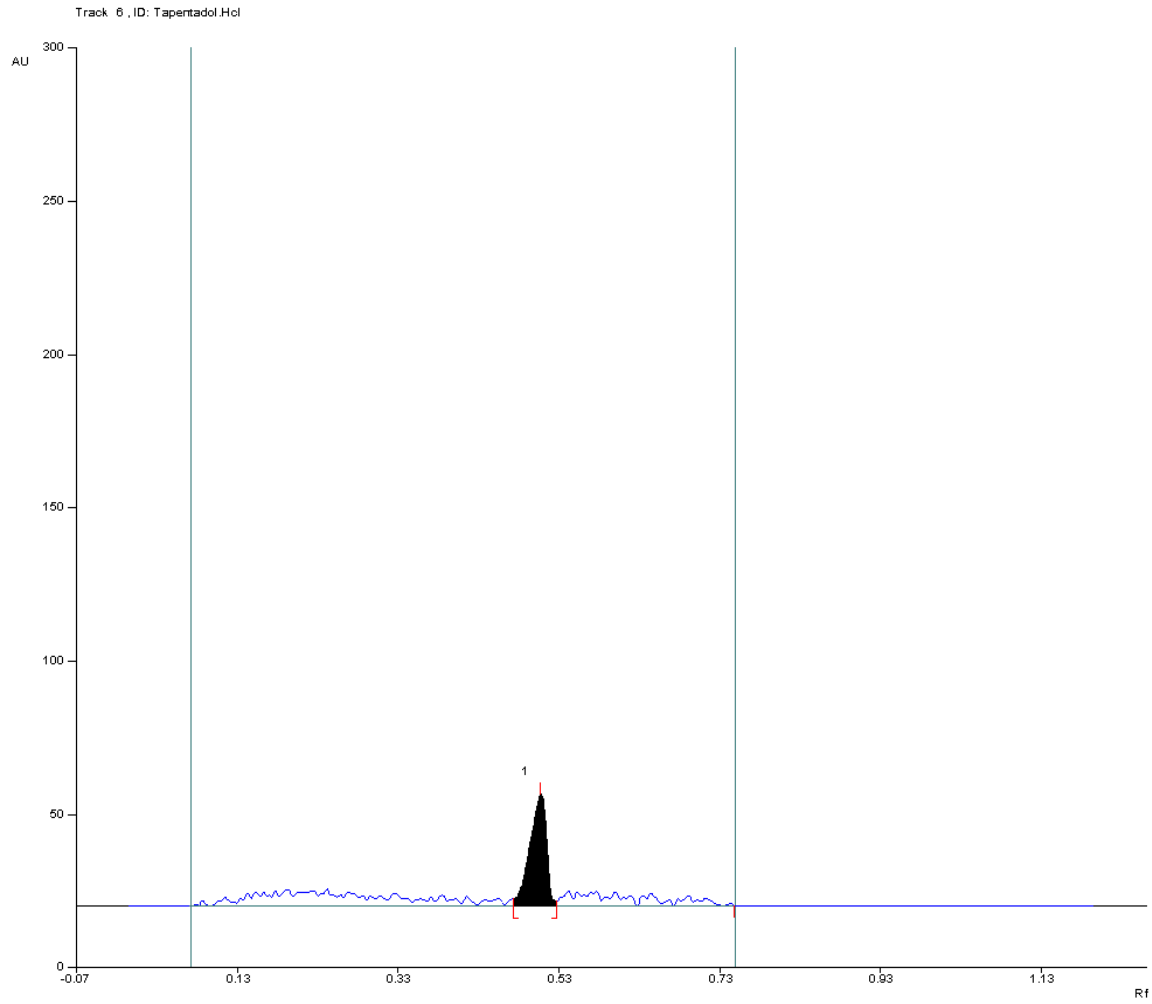
CHROMATOGRAM FOR FORMULATION ANALYSIS BY HPTLC REPEATABILITY 5



Peak	Start Rf	Start Height	Max Rf	Max Height	Max%	End Rf	End Height	Area	Area %
1	0.46	2.2	0.49	38.1	100%	0.52	1.9	549.3	100%

FIGURE – 33

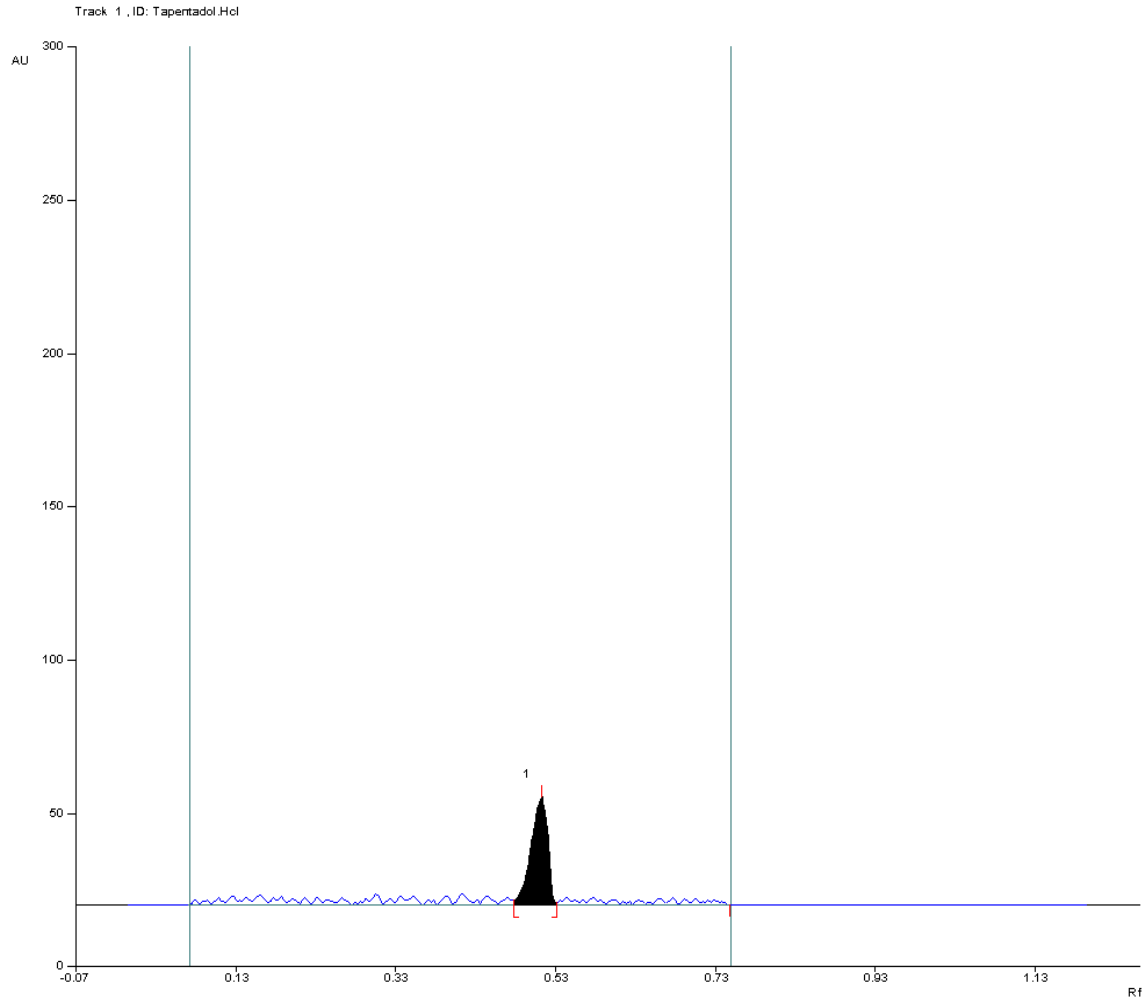
CHROMATOGRAM FOR FORMULATION ANALYSIS BY HPTLC REPEATABILITY 6



Peak	Start Rf	Start Height	Max Rf	Max Height	Max%	End Rf	End Height	Area	Area %
1	0.47	1.9	0.51	38.3	100%	0.54	1.5	550.4	100%

FIGURE - 34

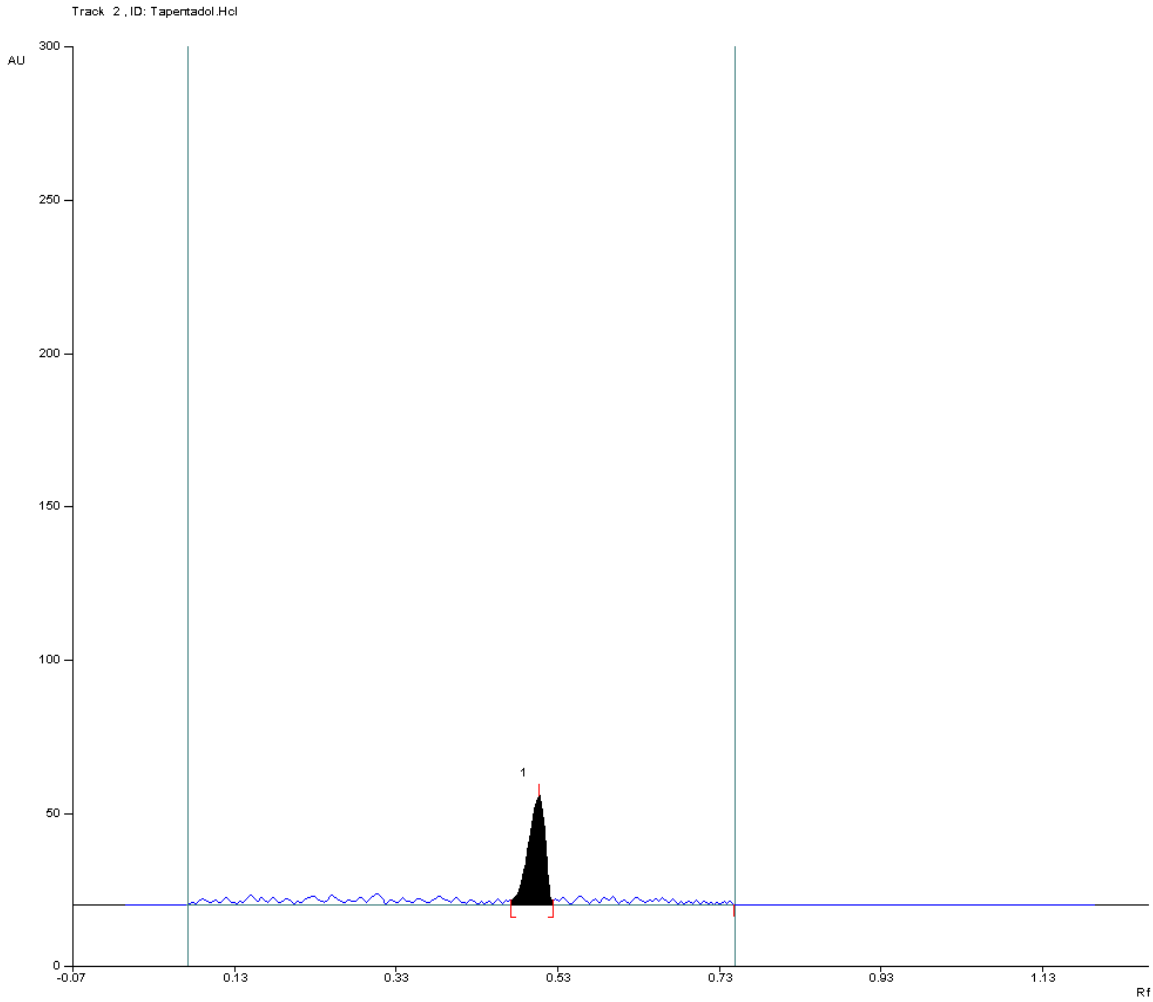
CHROMATOGRAM FOR FORMULATION ANALYSIS BY HPTLC INTRA DAY 1



Peak	Start Rf	Start Height	Max Rf	Max Height	Max%	End Rf	End Height	Area	Area %
1	0.45	2.1	0.50	36.8	100%	0.53	0.8	550.6	100%

FIGURE - 35

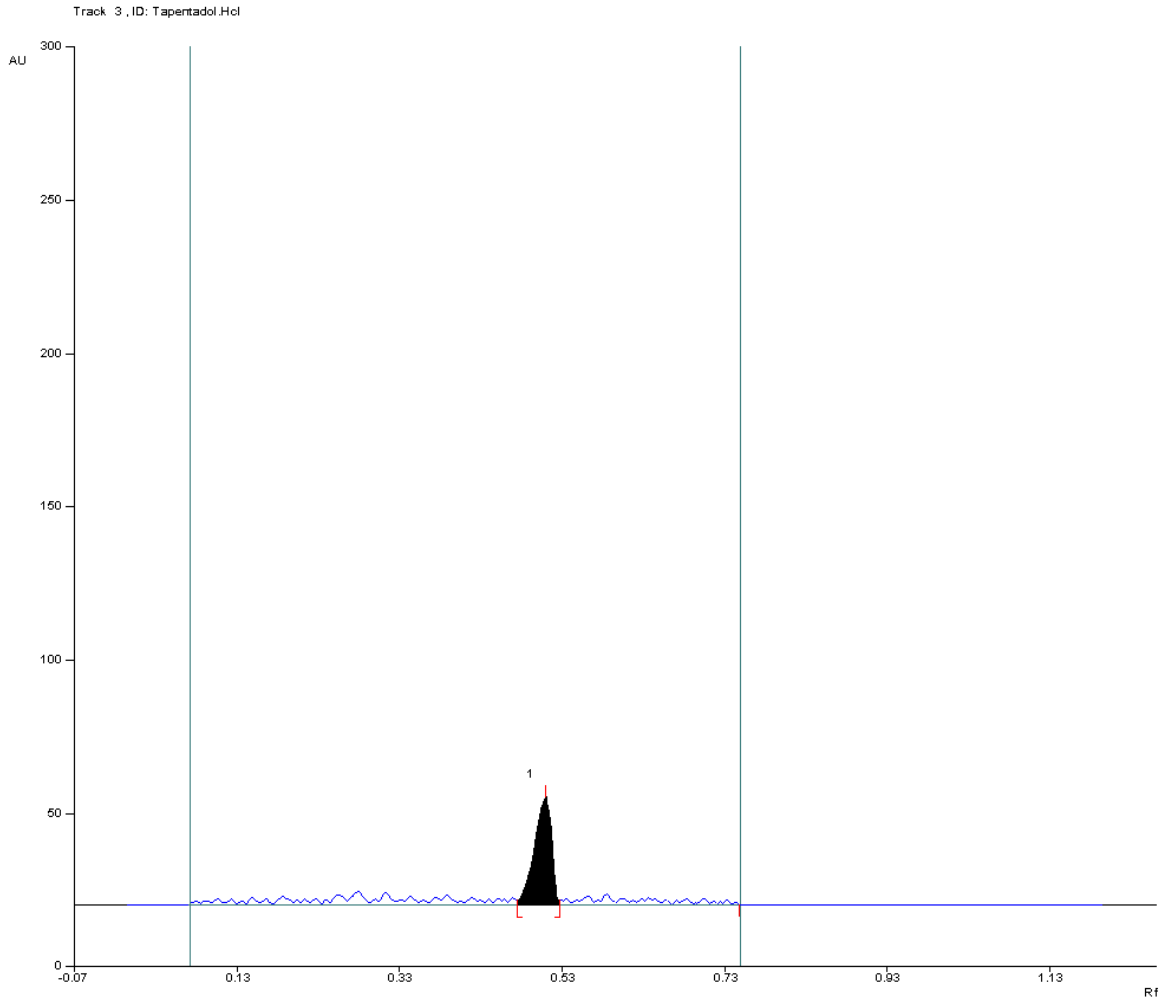
CHROMATOGRAM FOR FORMULATION ANALYSIS BY HPTLC INTRA DAY 2



Peak	Start Rf	Start Height	Max Rf	Max Height	Max%	End Rf	End Height	Area	Area %
1	0.45	2.2	0.50	37.5	100%	0.52	1.8	550.8	100%

FIGURE - 36

CHROMATOGRAM FOR FORMULATION ANALYSIS BY HPTLC INTRA DAY 3

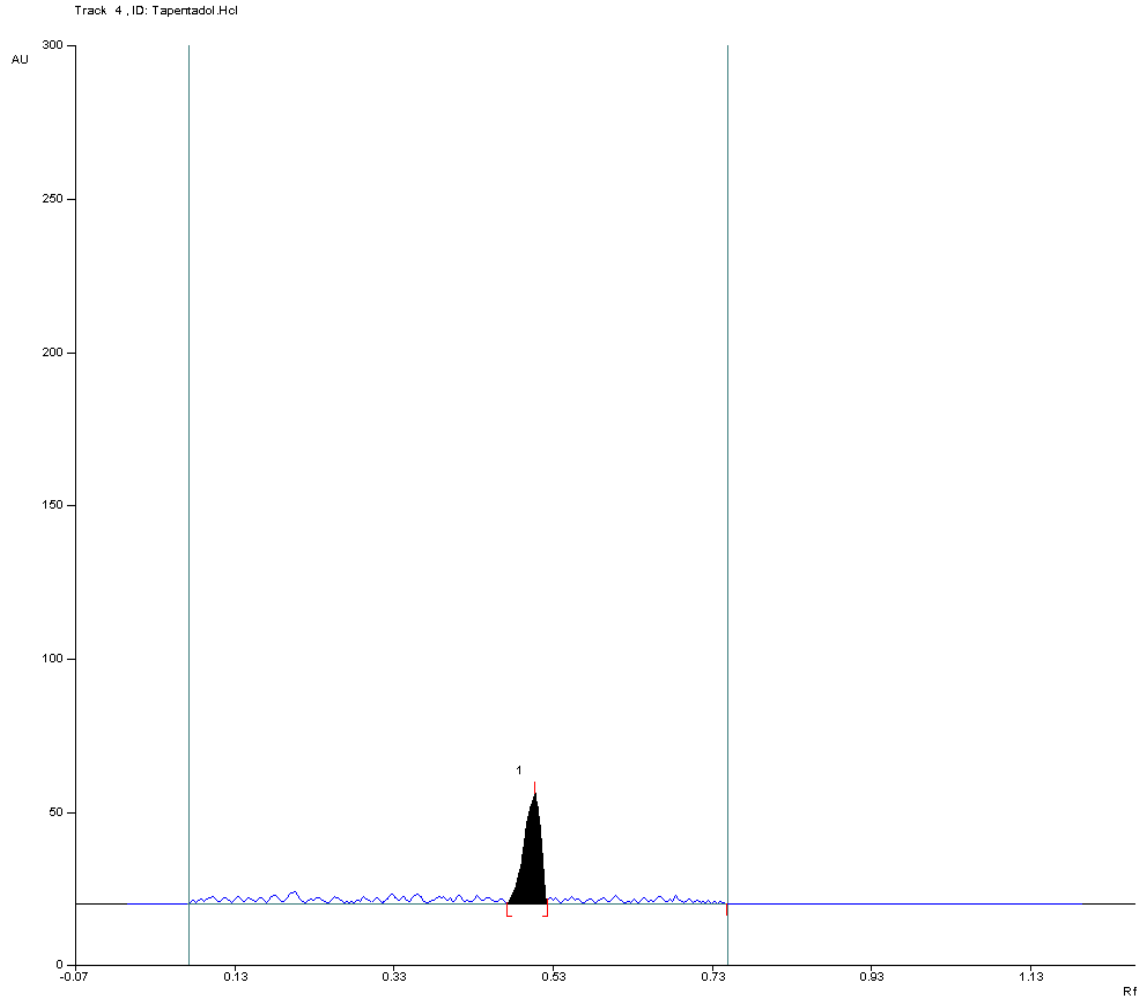


Peak	Start Rf	Start Height	Max Rf	Max Height	Max%	End Rf	End Height	Area	Area %
1	0.46	0.7	0.51	38.1	100%	0.53	2.0	553.8	100%

FIGURE - 37

CHROMATOGRAM FOR FORMULATION ANALYSIS BY HPTLC

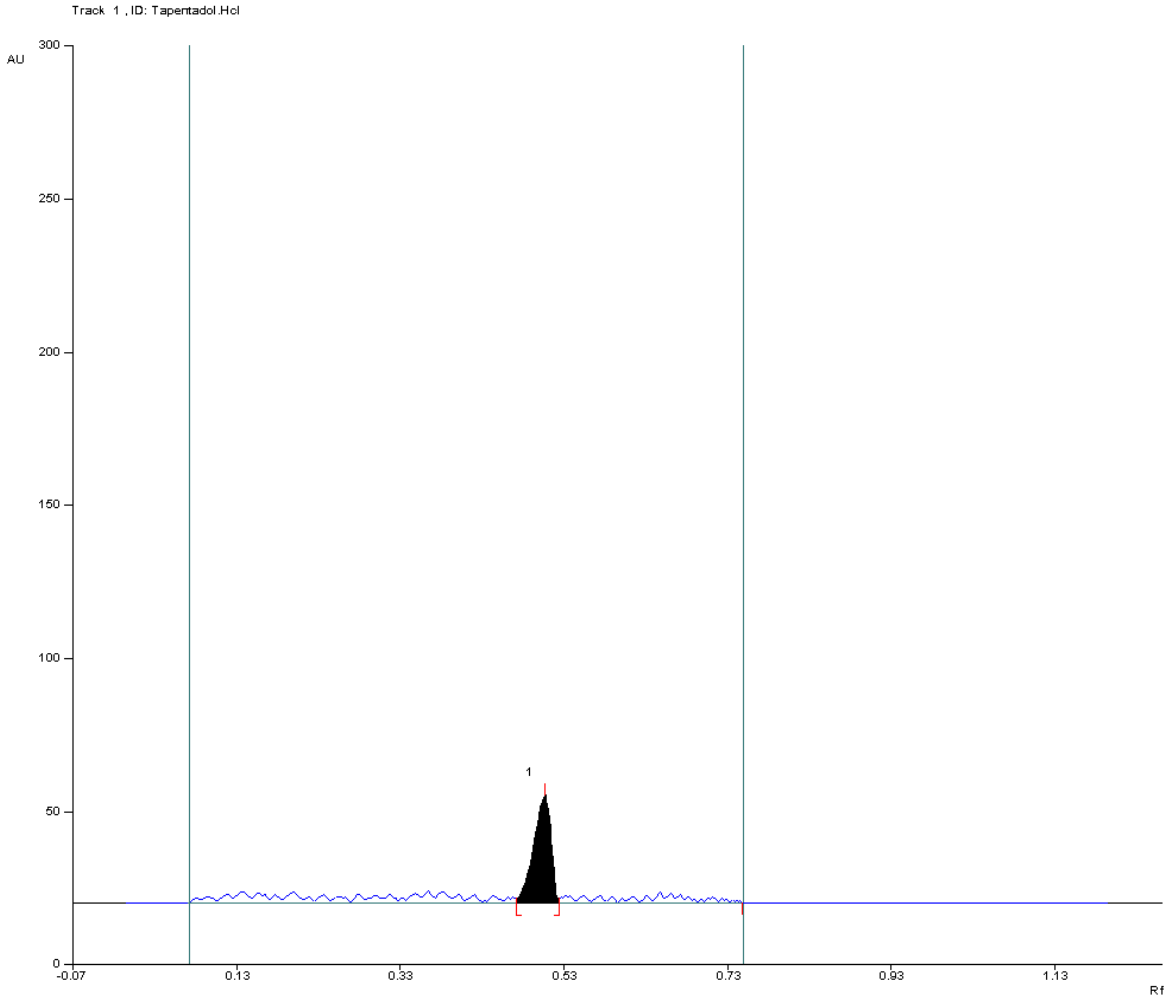
INTER DAY 1



Peak	Start Rf	Start Height	Max Rf	Max Height	Max%	End Rf	End Height	Area	Area %
1	0.45	0.5	0.49	38.2	100%	0.52	1.7	548.9	100%

FIGURE – 38

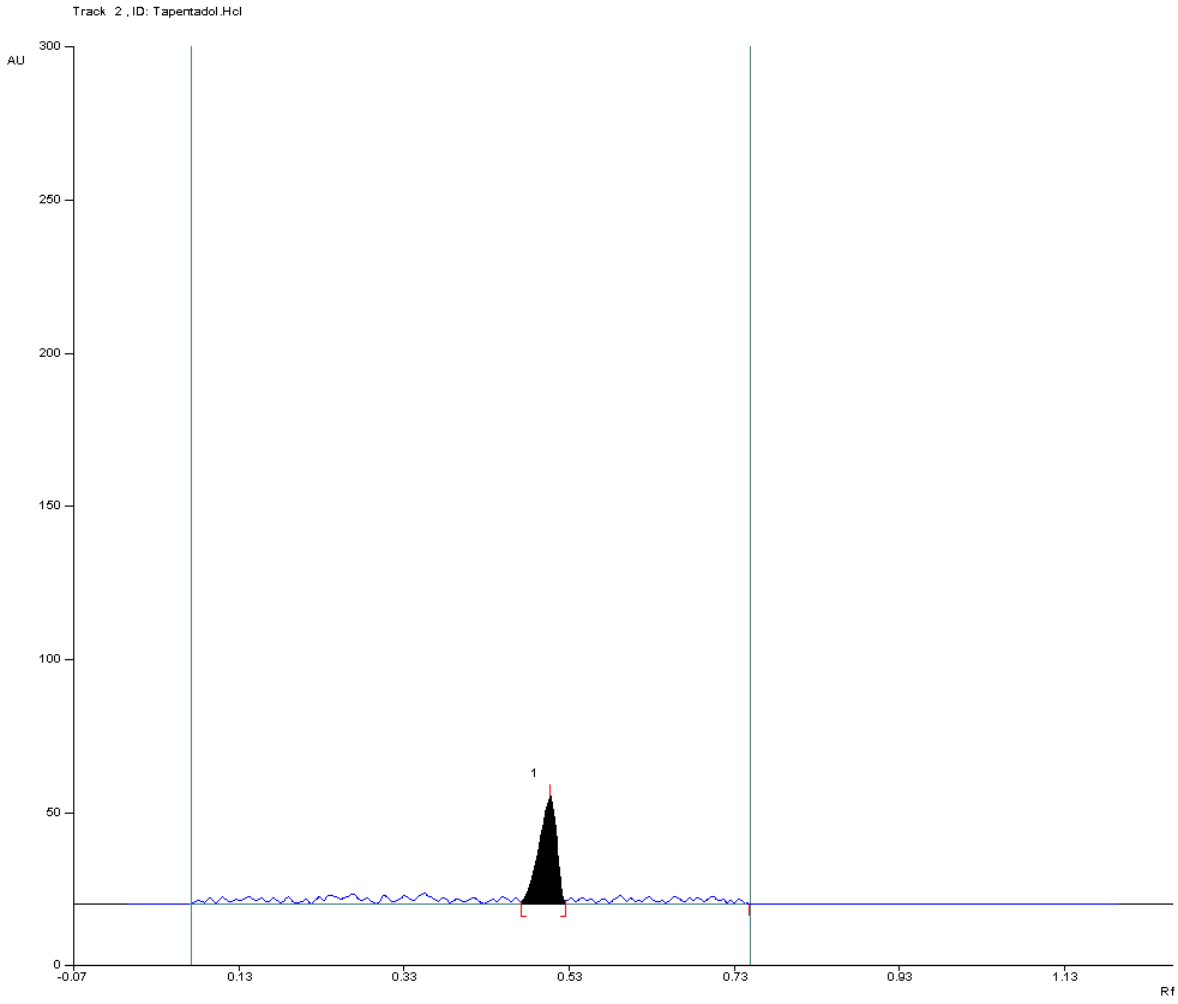
CHROMATOGRAM FOR FORMULATION ANALYSIS BY HPTLC INTER DAY 2



Peak	Start Rf	Start Height	Max Rf	Max Height	Max%	End Rf	End Height	Area	Area %
1	0.45	1.9	0.50	37.4	100%	0.52	2.1	551.4	100%

FIGURE – 39

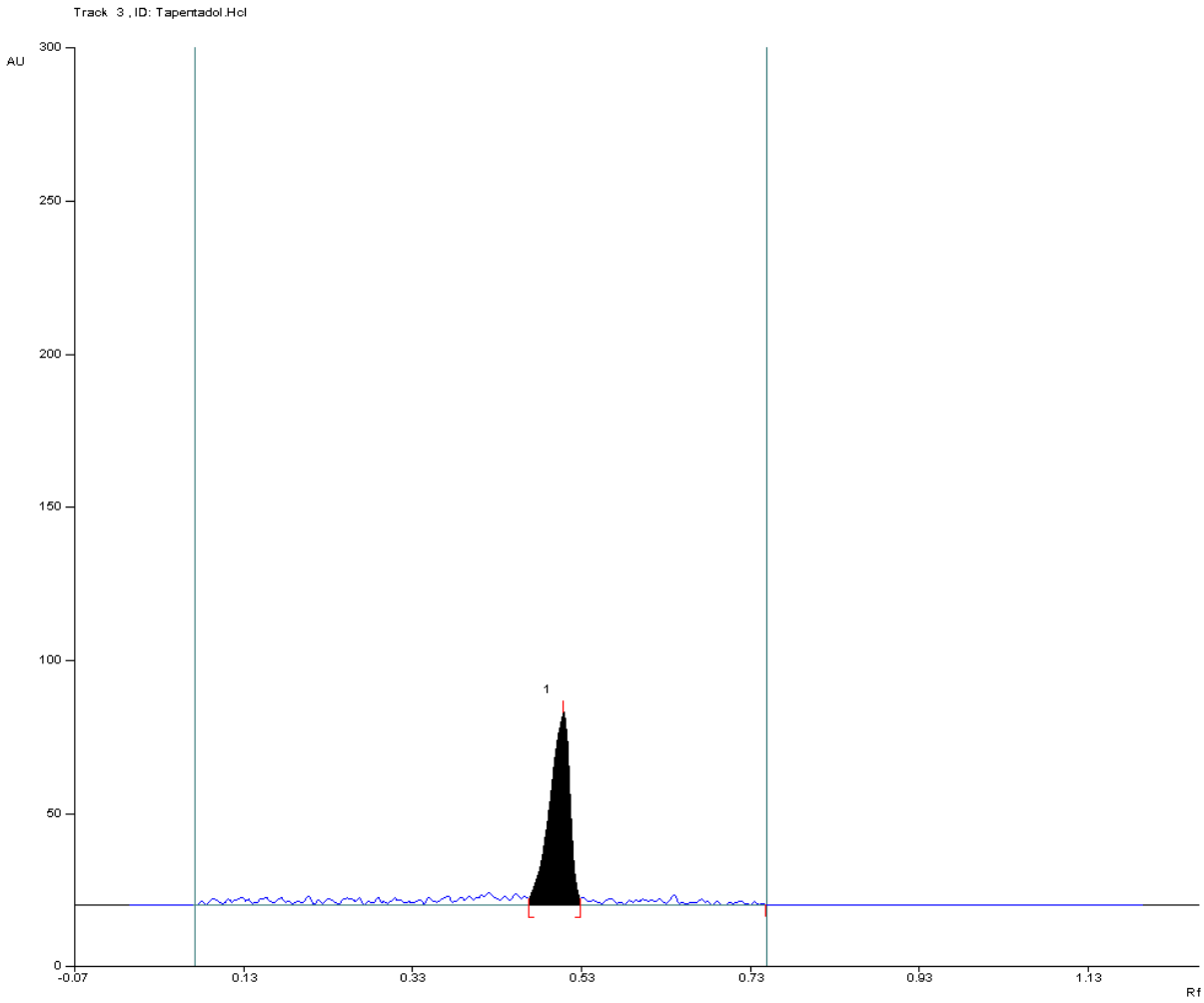
CHROMATOGRAM FOR FORMULATION ANALYSIS BY HPTLC INTER DAY 3



Peak	Start Rf	Start Height	Max Rf	Max Height	Max%	End Rf	End Height	Area	Area %
1	0.46	1.2	0.50	36.3	100%	0.53	1.8	550.6	100%

FIGURE – 40

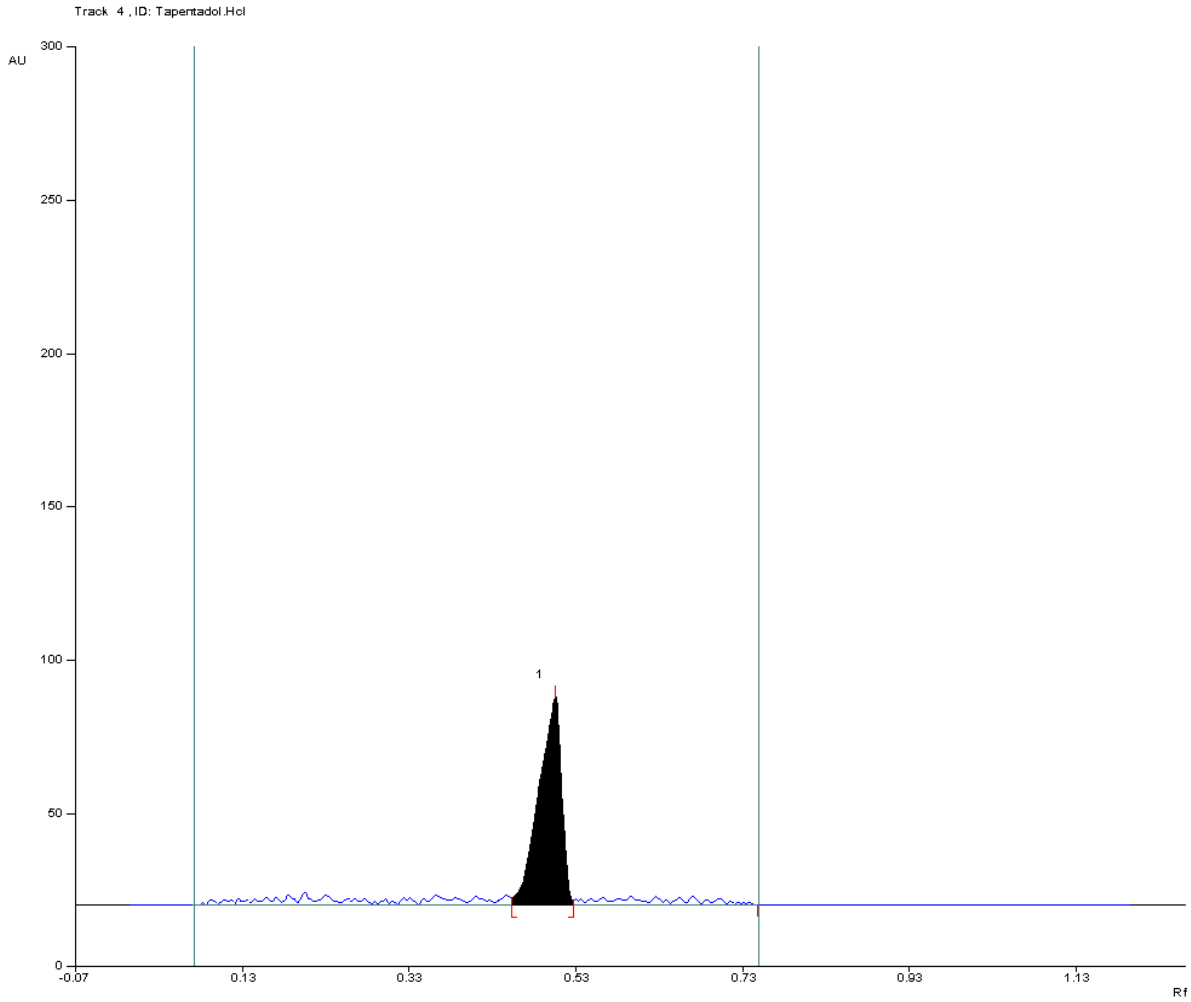
CHROMATOGRAM FOR RECOVERY ANALYSIS BY HPTLC (80%)



Peak	Start Rf	Start Height	Max Rf	Max Height	Max%	End Rf	End Height	Area	Area %
1	0.46	2.5	0.50	62.8	100%	0.54	2.3	978.2	100%

FIGURE - 41

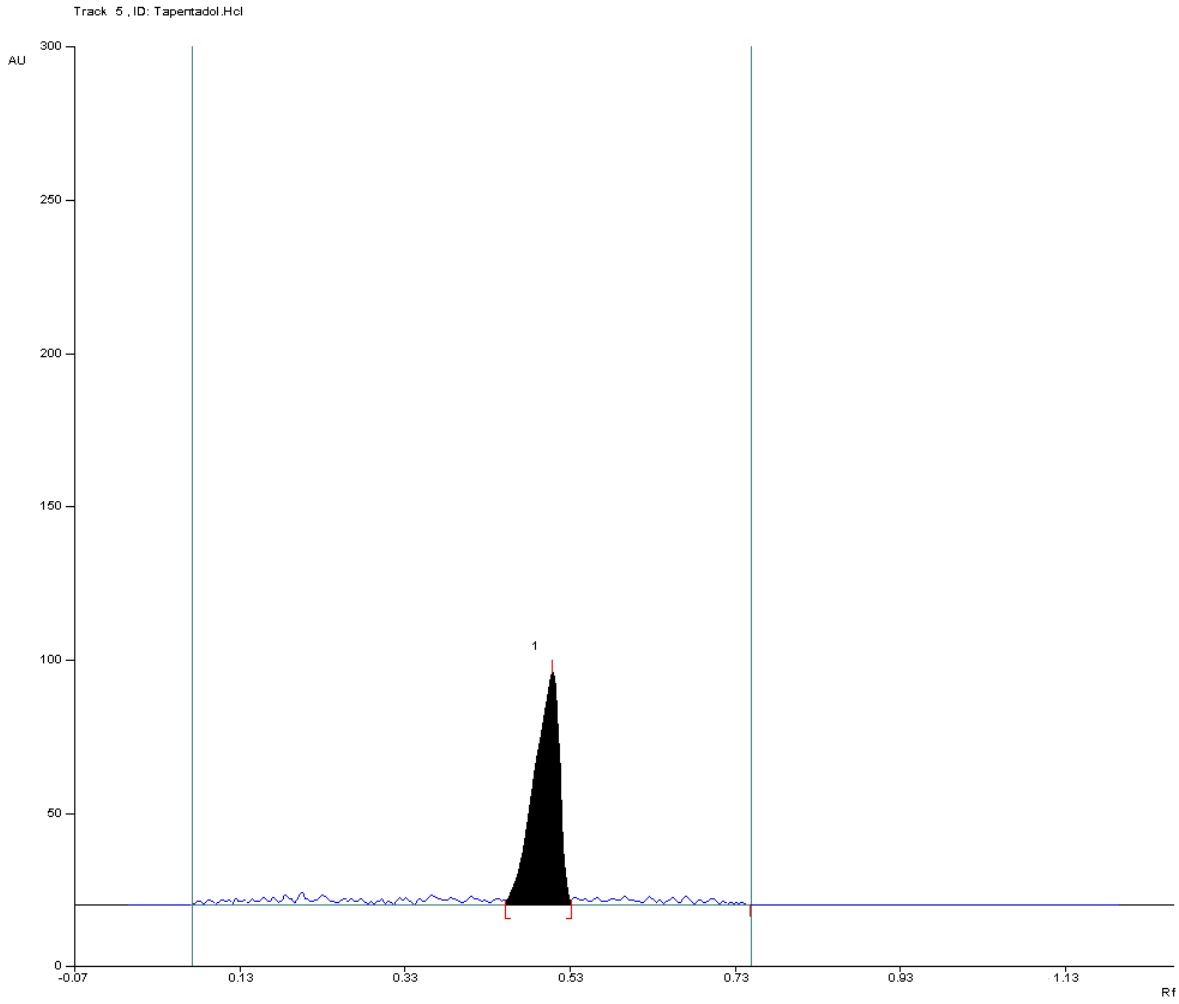
CHROMATOGRAM FOR RECOVERY ANALYSIS BY HPTLC (100%)



Peak	Start Rf	Start Height	Max Rf	Max Height	Max%	End Rf	End Height	Area	Area %
1	0.45	2.8	0.50	68.6	100%	0.54	2.1	1085.3	100%

FIGURE - 42

CHROMATOGRAM FOR RECOVERY ANALYSIS BY HPTLC (120%)



Peak	Start Rf	Start Height	Max Rf	Max Height	Max%	End Rf	End Height	Area	Area %
1	0.45	2.2	0.51	76.1	100%	0.54	2.1	1185.9	100%

Tables

TABLE – 1

SOLUBILITY PROFILE OF TAPENTADOL HYDROCHLORIDE IN POLAR AND NON-POLAR SOLVENTS

S. No	SOLVENTS	EXTENT OF SOLUBILITY	CATEGORY
1.	Distilled water	10mg in 10 µl	Very soluble
2.	Methanol	10 mg in 20 µl	Freely soluble
3.	Ethanol	10 mg in 40 µl	Freely soluble
4.	Acetonitrile	10 mg in 2.5 ml	Slightly soluble
5.	Di-methylFormamide	10 mg in 40.0 µl	Freely soluble
6.	Acetone	10 mg in more than 10.0ml	Practically insoluble
7.	acetic acid	10 mg in 20.0 µl	Freely soluble
8.	Ethyl acetate	10mg in 1.5ml	Slightly soluble
9.	Chloroform	10 mg in more than 10.0 ml	Practically insoluble
10.	Dichloromethane	10 mg in 6.0ml	Slightly soluble
11.	n- Butanol	10 mg in 300.0 µl	Soluble
12.	Pyridine	10 mg in 30.0 µl	Freely soluble
13.	Benzene	10 mg in more than 10.0ml	Practically insoluble
14.	Iso propyl alcohol	10 mg in 500.0 µl	Sparingly soluble
15.	Toluene	10 mg in 7.0ml	Slightly soluble
16.	Diethyl ether	10 mg in more than 10.0ml	Practically insoluble
17.	0.1N Hydrochloric Acid	10 mg in 10.0 µl	Very soluble
18.	0.1N Sodium Hydroxide	10 mg in 0.8 ml	Sparingly soluble
19.	pH-3.0(acid phthalate buffer)	10mg in 10.0 µl	Very soluble
20.	pH-5.0(neutralized phthalate buffer)	10mg in 10.0 µl	Very soluble
21.	pH-7.0(phosphate buffer)	10mg in 10.0 µl	Very soluble
22.	pH-9 (alkaline borate buffer)	10mg in 10.0 µl	Very soluble

TABLE – 2

OPTICAL CHARACTERISTICS OF TAPENTADOL HYDROCHLORIDE BY UV METHOD

PARAMETERS	VALUES*
λmax (nm)	272.5
Beer's law limit (µg/ ml)	10 - 60
Sandell's sensitivity ($\mu g/cm^2$/0.001 A.U)	0.014411
Molar absorptivity ($L\ mol^{-1}\ cm^{-1}$)	2.885×10^3
Correlation coefficient (r)	0.9999
Regression equation (y=mx+c)	Y = 0.0069x + 0.0019
Slope(m)	0.0069
Intercept(c)	0.0019
LOD (µg/ ml)	0.4255
LOQ (µg/ ml)	1.2893
Standard error	0.0002
Residual sum of squares	0.000020

*Mean of six observations

TABLE - 3

QUANTIFICATION OF RAW MATERIAL BY UV METHOD

Drug	Sample No.	Amount found (µg/ ml)*	Percentage Obtained* (%)	Mean (%)	SD	% RSD	SE	CI
TAP	1	19.6014	98.01	98.21	0.6159	0.6271	0.0171	97.56 to 98.86
	2	19.5174	97.59					
	3	19.8752	99.38					
	4	19.6446	98.22					
	5	19.5773	97.89					
	6	19.6350	98.18					

*Mean of six observations

TABLE - 4

QUANTIFICATION OF FORMULATION TAPOL-100 BY UV METHOD

Drug	Sample No.	Labeled amount (mg/tab)	Amount found (mg/tab)*	Percentage Obtained* %(w/v)	Mean (%)	SD	% RSD	SE	CI
TAP	1	100.0mg	100.52	100.52	100.56	0.3153	0.3136	0.0088	100.23 to 100.89
	2	100.0mg	100.14	100.14					
	3	100.0mg	100.24	100.24					
	4	100.0mg	100.92	100.92					
	5	100.0mg	100.83	100.83					
	6	100.0mg	100.68	100.68					

*Mean of six observations

TABLE - 5

INTRADAY ANALYSIS OF FORMULATION TAPOL-100 BY UV METHOD

Drug	Sample No	Labeled amount (mg/tab)	Amount found (mg/tab)*	Percentage Obtained*	Mean (%)	SD	% RSD	SE	CI
TAP	1	100	100.05	100.05	99.91	0.2779	0.2781	0.0309	99.22 to 100.60
	2	100	100.09	100.09					
	3	100	99.59	99.59					

*Mean of six observations

TABLE - 6

INTERDAY ANALYSIS OF FORMULATION TAPOL-100 BY UV METHOD

Drug	Sample No	Labeled amount (mg/tab)	Amount found (mg/tab)*	Percentage Obtained*	Mean (%)	SD	% RSD	SE	CI
TAP	1	100	100.63	100.63	100.27	0.3504	0.3495	0.0389	99.40 to 101.14
	2	100	99.93	99.93					
	3	100	100.25	100.25					

*Mean of six observations

TABLE – 7

RUGGEDNESS STUDY BY UV METHOD

(DIFFERENT ANALYSTS)

Drug	Condition	Sample No	Labeled Amount (mg/tab)	Amount found (mg/tab)*	Percentage Obtained*	Mean (%)	SD	% RSD	SE	CI
TAP	Analyst 1	1	100	100.38	100.38	100.15	0.3674	0.3669	0.0102	99.76 to 100.54
		2	100	100.46	100.46					
		3	100	99.49	99.49					
		4	100	100.27	100.27					
		5	100	100.34	100.34					
		6	100	99.95	99.95					
TAP	Analyst 2	1	100	100.92	100.92	100.52	0.4000	0.398	0.0111	100.10 to 100.94
		2	100	99.86	99.86					
		3	100	100.93	100.93					
		4	100	100.54	100.54					
		5	100	100.52	100.52					
		6	100	100.33	100.33					

*Mean of six observations

TABLE – 8

RUGGEDNESS STUDY BY UV METHOD

(DIFFERENT INSTRUMENTS)

Drug	Condition	Sample No	Labeled amount (mg/tab)	Amount found (mg/tab)*	Percentage Obtained*	Mean (%)	SD	% RSD	SE	CI
TAP	Instrument 1	1 2 3 4 5 6	100 100 100 100 100 100	100.40 100.25 100.05 100.09 100.63 99.59	100.40 100.25 100.05 100.09 100.63 99.59	100.17	0.3544	0.3538	0.0980	99.80 to 100.54
TAP	Instrument 2	1 2 3 4 5 6	100 100 100 100 100 100	100.63 100.99 99.39 98.42 100.01 99.82	100.63 100.99 99.39 98.42 100.01 99.82	100.04	0.8996	0.8992	0.0250	98.10 to 100.98

*Mean of six observations

TABLE – 9

RECOVERY ANALYSIS OF FORMULATION TAPOL -100 BY UV METHOD

Drug	Sample No	Amount present (µg/ml)	Amount added (µg/ml)	Amount found* (µg/ml)	Amount recovered (µg/ml)	% Recovered	SD	% RSD	SE	CI
TAP	1	20.1803	15.5207	35.6907	15.5104	99.93	0.8759	0.8705	0.0973	98.45 to 102.81
	2	20.1803	15.5159	35.9453	15.7650	101.61				
	3	20.1803	15.5495	35.7819	15.6016	100.34				
					Mean	100.63				
TAP	1	20.1803	19.5005	39.6248	19.4445	99.71	0.9253	0.9205	0.1028	98.22 to 102.82
	2	20.1803	19.4782	39.9562	19.7759	101.53				
	3	20.1803	19.4169	39.6609	19.4806	100.33				
					Mean	100.52				
TAP	1	20.1803	23.5857	44.0297	23.8494	101.11	0.5640	0.5614	0.0627	99.05 to 101.86
	2	20.1803	23.5617	43.7822	23.6019	100.17				
	3	20.1803	23.7203	43.9239	23.7436	100.10				
					Mean	100.46				

*Mean of three observations

TABLE – 10

OPTICAL CHARACTERISTICS OF TAPENTADOL HYDROCHLORIDE BY DIFFERENCE SPECTROPHOTOMETRIC METHOD

PARAMETERS	VALUES*
Wave length maxima	290 nm
Wave length minima	269.5 nm
Beer's law limit (µg/ ml)	3 - 18
Sandell's sensitivity ($\mu g/cm^2$/0.001 A.U)	0.2452
Molar absorptivity ($L\ mol^{-1}\ cm^{-1}$)	1.6858×10^3
Correlation coefficient (r)	0.9999
Regression equation (y=mx+c)	Y = 0.0136x + (-0.0001)
Slope(m)	0.0136
Intercept(c)	-0.0001
LOD (µg/ ml)	0.6689
LOQ (µg/ ml)	2.0270
Standard error	0.0002
Residual sum of squares	0.0000045

*Mean of six observations

TABLE – 11

QUANTIFICATION OF RAW MATERIAL BY DIFFERENCE SPECTROPHOTOMETRIC METHOD

Drug	Sample No.	Amount found (µg/ml)*	Percentage Obtained*	Mean (%)	SD	% RSD	SE	CI
TAP	1	12.0746	100.62	100.84	0.2562	0.2540	0.0071	100.57 to 101.11
	2	12.1064	100.89					
	3	12.0966	100.80					
	4	12.0819	100.68					
	5	12.0893	100.74					
	6	12.1592	101.33					

*Mean of six observations

TABLE – 12

QUANTIFICATION OF FORMULATION TAPOL-100 BY DIFFERENCE SPECTROPHOTOMETRIC METHOD

Drug	Sample No	Labeled amount (mg/tab)	Amount found (mg/tab)*	Percentage Obtained*	Mean (%)	SD	% RSD	SE	CI
TAP	1	100	101.4080	101.41	100.60	0.5872	0.5837	0.0163	99.98 to 101.21
	2	100	99.7941	99.79					
	3	100	101.1525	101.15					
	4	100	100.3758	100.38					
	5	100	100.5083	100.51					
	6	100	100.3700	100.37					

*Mean of six observations

TABLE – 13

INTRADAY ANALYSIS OF FORMULATION TAPOL-100 BY DIFFERENCE SPECTROPHOTOMETRIC METHOD

Drug	Sample No	Labeled amount (mg/tab)	Amount found (mg/tab)*	Percentage Obtained*	Mean (%)	SD	% RSD	SE	CI
TAP	1	100	99.8550	99.86	99.67	0.3177	0.3188	0.0353	98.88 to 100.46
	2	100	99.8350	99.84					
	3	100	99.3033	99.30					

*Mean of six observations

TABLE –14

INTERDAY ANALYSIS OF FORMULATION TAPOL-100 BY DIFFERENCE SPECTROPHOTOMETRIC METHOD

Drug	Sample No	Labeled amount (mg/tab)	Amount found (mg/tab)*	Percentage Obtained*	Mean (%)	SD	% RSD	SE	CI
TAP	1	100	100.0967	100.10	99.85	0.2663	0.2667	0.0296	99.19 to 100.51
	2	100	99.8750	99.88					
	3	100	99.5683	99.57					

*Mean of six observations

TABLE –15

RUGGEDNESS STUDY BY DIFFERENCE SPECTROPHOTOMETRIC METHOD

Drug	Condition	Sample No	Labeled Amount (mg/tab)	Amount found (mg/tab)*	Percentage Obtained*	Mean (%)	SD	% RSD	SE	CI
TAP	Analyst 1	1	100	99.8508	99.85	99.85	0.2361	0.2364	0.0066	99.60 to 100.10
		2	100	99.8958	99.90					
		3	100	100.1917	100.19					
		4	100	99.9875	99.99					
		5	100	99.5683	99.57					
		6	100	99.5958	99.60					
TAP	Analyst 2	1	100	100.5296	100.53	100.10	0.4070	0.4066	0.0113	99.67 to 100.53
		2	100	100.2951	100.30					
		3	100	100.0993	100.10					
		4	100	99.4164	99.42					
		5	100	100.3769	100.38					
		6	100	99.8456	99.85					

*Mean of six observations

TABLE – 16

RUGGEDNESS STUDY BY DIFFERENCE SPECTROPHOTOMETRIC METHOD

Drug	Condition	Sample No	Labeled amount (mg/tab)	Amount found (mg/tab)*	Percentage Obtained*	Mean (%)	SD	% RSD	SE	CI
TAP	Instrument 1	1 2 3 4 5 6	100 100 100 100 100 100	101.4083 101.1525 100.5083 99.8550 99.3033 100.0967	101.41 101.15 100.51 99.86 99.30 100.10	100.39	0.7219	0.7191	0.0201	99.63 to 101.15
TAP	Instrument 2	1 2 3 4 5 6	100 100 100 100 100 100	100.4067 100.0249 100.0822 100.1314 101.2742 100.3680	100.41 100.03 100.08 100.13 101.27 100.37	100.38	0.4637	0.4620	0.0129	99.89 to 100.87

*Mean of six observations

TABLE – 17

RECOVERY ANALYSIS OF FORMULATION TAPOL-100 BY DIFFERENCE SPECTROPHOTOMETRIC METHOD

Drug	Sample No	Amount present (µg/ml)	Amount added (µg/ml)	Amount found* (µg/ml)	Amount recovered (µg/ml)	% Recovered	SD	% RSD	SE	CI
TAP	1	6.0506	4.4042	10.3879	4.3373	98.48	0.3807	0.3851	0.0423	97.90 to 99.80
	2	6.0506	4.4153	10.4137	4.3631	98.82				
	3	6.0506	4.3859	10.3765	4.3259	99.24				
					Mean	98.85				
TAP	1	6.0506	5.8800	11.8700	5.8194	98.97	0.1242	0.1254	0.0138	98.73 to 99.35
	2	6.0506	5.9724	11.9740	5.9234	99.18				
	3	6.0506	5.8901	11.8797	5.8291	98.96				
					Mean	99.04				
TAP	1	6.0506	7.3517	13.2640	7.2134	98.12	0.3345	0.3401	0.0372	97.54 to 99.20
	2	6.0506	7.3237	13.2455	7.1949	98.24				
	3	6.0506	7.2267	13.1867	7.1361	98.75				
					Mean	98.37				

*Mean of three observations

TABLE – 18

OPTICAL CHARACTERISTICS OF TAPENTADOL HYDROCHLORIDE BY COLORIMETRIC METHOD-I

PARAMETERS	VALUES*
λmax (nm)	514
Beer's law limit (µg/ ml)	2-24
Sandell's sensitivity (µg/cm^2/0.001 A.U)	0.02044
Molar absorptivity	1.1366 $\times 10^4$
Correlation coefficient (r)	0.9991
Regression equation (y = mx + c)	Y = 0.0490x + 0.0237
Slope (m)	0.0490
Intercept (c)	0.0237
LOD (µg/ ml)	1.0473
LOQ (µg/ ml)	3.1736
Standard error	0.0008
Residual sum of squares	0.001028

*Mean of six observations

TABLE –19

QUANTIFICATION OF RAW MATERIAL BY COLORIMETRIC METHOD-I

Drug	Sample No.	Amount found (µg/ml)*	Percentage Obtained*	Mean (%)	SD	% RSD	SE	CI
TAP	1	10.0348	100.35	101.08	0.7682	0.7600	0.0213	100.27 to 101.89
	2	10.0660	100.66					
	3	10.1216	101.22					
	4	10.2138	102.14					
	5	10.1780	101.78					
	6	10.0328	100.33					

*Mean of six observations

TABLE – 20

QUANTIFICATION OF FORMULATION TAPOL-100 BY COLORIMETRIC METHOD-I

Drug	Sample No	Labeled amount (mg/tab)	Amount found (mg/tab)*	Percentage Obtained*	Mean (%)	SD	% RSD	SE	CI
TAP	1	100	99.5420	99.54	100.05	0.3318	0.3317	0.0092	99.70 to 100.40
	2	100	100.0490	100.05					
	3	100	99.7708	99.77					
	4	100	100.3673	100.37					
	5	100	100.3400	100.34					
	6	100	100.2100	100.21					

*Mean of six observations

TABLE – 21

INTRADAY ANALYSIS OF FORMULATION TAPOL-100 BY COLORIMETRIC METHOD-I

Drug	Sample No	Labeled amount (mg/tab)	Amount found (mg/tab) *	Percentage Obtained*	Mean (%)	SD	% RSD	SE	CI
TAP	1	100	99.6980	99.70	99.73	0.4806	0.4819	0.0534	99.54 To 100.92
	2	100	100.2160	100.22					
	3	100	99.2619	99.26					

*Mean of three observations

TABLE – 22

INTERDAY ANALYSIS OF FORMULATION TAPOL-100 BY COLORIMETRIC METHOD-I

Drug	Sample No	Labeled amount (mg/tab)	Amount found (mg/tab)*	Percentage Obtained*	Mean (%)	SD	% RSD	SE	CI
TAP	1	100	100.0250	100.03	99.99	0.2227	0.2227	0.0247	99.44 to 100.54
	2	100	99.7473	99.75					
	3	100	100.1850	100.19					

*Mean of three observations

TABLE – 23

RUGGEDNESS STUDY BY COLORIMETRIC METHOD-I
(DIFFERENT ANALYSTS)

Drug	Condition	Sample No	Labeled Amount (mg/tab)	Amount found (mg/tab)*	Percentage Obtained*	Mean (%)	SD	% RSD	SE	CI
TAP	Analyst 1	1	100	100.0490	100.05	100.18	0.1250	0.1247	0.0035	100.05 to 100.31
		2	100	100.3673	100.37					
		3	100	100.2100	100.21					
		4	100	100.2160	100.22					
		5	100	100.0250	100.03					
		6	100	100.1850	100.19					
TAP	Analyst 2	1	100	100.0968	100.10	100.65	0.3646	0.3622	0.0101	100.27 to 101.03
		2	100	100.6480	100.65					
		3	100	100.4330	100.43					
		4	100	101.0460	101.05					
		5	100	101.0360	101.04					
		6	100	100.6100	100.61					

*Mean of six observations

TABLE – 24

RUGGEDNESS STUDY BY COLORIMETRIC METHOD-I

(DIFFERENT INSTRUMENTS)

Drug	Condition	Sample No	Labeled amount (mg/tab)	Amount found (mg/tab)*	Percentage Obtained*	Mean (%)	SD	% RSD	SE	CI
TAP	Instrument 1	1	100	99.5420	99.54	99.73	0.3554	0.3564	0.0099	99.36 to 100.10
		2	100	99.7701	99.77					
		3	100	100.3400	100.34					
		4	100	99.6980	99.70					
		5	100	99.2619	99.26					
		6	100	99.7400	99.74					
TAP	Instrument 2	1	100	100.3580	100.36	101.39	0.7671	0.7566	0.0213	100.59 to 102.20
		2	100	101.5530	101.55					
		3	100	102.3400	102.34					
		4	100	102.1480	102.15					
		5	100	101.0530	101.05					
		6	100	100.8760	100.88					

*Mean of six observations

TABLE – 25

RECOVERY ANALYSIS OF FORMULATION TAPOL-100 BY COLORIMETRIC METHOD-I

Drug	Sample No	Amount present (µg/ml)	Amount added (µg/ml)	Amount found* (µg/ml)	Amount recovered (µg/ml)	% Recovered	SD	% RSD	SE	CI
TAP	1	10.0038	7.9759	18.1384	8.1346	101.99	0.6331	0.6215	0.0703	100.30 to 103.44
	2	10.0038	7.9449	18.1426	8.1388	102.44				
	3	10.0038	8.0419	18.1419	8.1381	101.19				
					Mean	101.87				
TAP	1	10.0038	9.9722	20.1483	10.1445	101.73	0.7447	0.7347	0.0828	99.52 to 103.22
	2	10.0038	10.0426	20.2334	10.2296	101.86				
	3	10.0038	10.1632	20.2187	10.2149	100.51				
					Mean	101.37				
TAP	1	10.0038	12.1469	22.3246	12.3208	101.43	0.1700	0.1679	0.0188	100.81 to 101.65
	2	10.0038	12.1261	22.2682	12.2644	101.14				
	3	10.0038	12.1302	22.2712	12.2674	101.13				
					Mean	101.23				

*Mean of three observations

TABLE – 26

OPTICAL CHARACTERISTICS OF TAPENTADOL.HCL BY COLORIMETRIC METHOD-II

PARAMETERS	VALUES*
λmax (nm)	737
Beer's law limit (µg/ ml)	1-10
Sandell's sensitivity ($\mu g/cm^2$/0.001 A.U)	0.0063
Molar absorptivity	4.1435×10^4
Correlation coefficient (r)	0.9997
Regression equation (y = mx + c)	Y = 0.1594x + 0.0133
Slope (m)	0.1594
Intercept (c)	0.0133
LOD (µg/ ml)	0.1605
LOQ (µg/ ml)	0.4865
Standard error	0.0009
Residual sum of squares	0.000711

*Mean of six observations

TABLE –27

QUANTIFICATION OF RAW MATERIAL BY COLORIMETRIC METHOD-II

Drug	Sample No.	Amount found (µg/ml)*	Percentage Obtained*	Mean (%)	SD	% RSD	SE	CI
TAP	1	4.0084	100.21	100.16	0.2605	0.2601	0.0072	99.89 to 100.43
	2	4.0128	100.32					
	3	4.0168	100.42					
	4	4.0107	100.27					
	5	3.9955	99.89					
	6	3.9904	99.76					

*Mean of six observations

TABLE – 28

QUANTIFICATION OF FORMULATION TAPOL-100 BY COLORIMETRIC METHOD-II

Drug	Sample No	Labeled amount (mg/tab)	Amount found (mg/tab)*	Percentage Obtained*	Mean (%)	SD	% RSD	SE	CI
TAP	1	100	98.5923	98.59	100.13	0.8343	0.8332	0.0232	99.25 to 101.01
	2	100	99.9150	99.92					
	3	100	100.1790	100.18					
	4	100	100.5816	100.58					
	5	100	100.4980	100.5					
	6	100	100.9789	100.98					

*Mean of six observations

TABLE – 29

INTRADAY ANALYSIS OF FORMULATION TAPOL-100 BY COLORIMETRIC METHOD-II

Drug	Sample No	Labeled amount (mg/tab)	Amount found (mg/tab)*	Percentage Obtained*	Mean (%)	SD	% RSD	SE	CI
TAP	1	100	100.0614	100.06	99.92	0.2570	0.2572	0.0286	99.28 to 100.56
	2	100	99.6222	99.62					
	3	100	100.066	100.07					

*Mean of six observations

TABLE – 30

INTERDAY ANALYSIS OF FORMULATION TAPOL-100 BY COLORIMETRIC METHOD-II

Drug	Sample No	Labeled amount (mg/tab)	Amount found (mg/tab)*	Percentage Obtained*	Mean (%)	SD	% RSD	SE	CI
TAP	1	100	99.6693	99.67	100.35	0.5935	0.5919	0.0659	98.88 to 101.82
	2	100	100.6496	100.65					
	3	100	100.7437	100.74					

*Mean of six observations

TABLE – 31

RUGGEDNESS STUDY BY COLORIMETRIC METHOD-II

(DIFFERENT ANALYSTS)

Drug	Condition	Sample No	Labeled Amount (mg/tab)	Amount found (mg/tab)*	Percentage Obtained*	Mean (%)	SD	% RSD	SE	CI
TAP	Analyst 1	1	100	99.9150	99.92	100.23	0.2825	0.2819	0.0078	99.93 to 100.53
		2	100	100.1790	100.18					
		3	100	100.4980	100.50					
		4	100	100.0614	100.07					
		5	100	100.0666	100.07					
		6	100	100.6496	100.65					
TAP	Analyst 2	1	100	100.0144	100.01	99.73	0.7919	0.7941	0.0220	98.90 to 100.56
		2	100	100.3777	100.38					
		3	100	99.1962	99.20					
		4	100	98.3623	98.36					
		5	100	100.1215	100.12					
		6	100	100.2967	100.30					

*Mean of six observations

TABLE – 32

RUGGEDNESS STUDY BY COLORIMETRIC METHOD –II

(DIFFERENT INSTRUMENTS)

Drug	Condition	Sample No	Labeled amount (mg/tab)	Amount found (mg/tab)*	Percentage Obtained*	Mean (%)	SD	% RSD	SE	CI
TAP	Instrument 1	1	100	98.5923	98.59	100.01	0.7350	0.7349	0.0204	99.24 to 100.78
		2	100	100.1790	100.18					
		3	100	100.4980	100.50					
		4	100	100.0614	100.06					
		5	100	100.0666	100.07					
		6	100	100.6496	100.65					
TAP	Instrument 2	1	100	101.5645	101.57	100.99	0.3188	0.3157	0.0089	100.66 to 101.32
		2	100	100.8979	100.90					
		3	100	100.9685	100.97					
		4	100	100.6260	100.63					
		5	100	101.0600	101.06					
		6	100	100.8169	100.82					

*Mean of six observations

TABLE – 33

RECOVERY ANALYSIS OF FORMULATION - TAPOL-100 BY COLORIMETRIC METHOD-II

Drug	Sample No	Amount present (µg/ml)	Amount added (µg/ml)	Amount found* (µg/ml)	Amount recovered (µg/ml)	% Recovered	SD	% RSD	SE	CI
TAP	1	4.0050	3.2006	7.2550	3.2500	101.37	0.5436	0.5396	0.0604	99.41 to 102.10
	2	4.0050	3.2232	7.2408	3.2358	100.39				
	3	4.0050	3.2119	7.2324	3.2274	100.48				
					Mean	100.75				
TAP	1	4.0050	4.0183	8.0766	4.0716	101.33	1.1010	1.0982	0.1222	97.53 to 103.00
	2	4.0050	4.0812	8.0505	4.0455	99.13				
	3	4.0050	4.0066	8.0241	4.0191	100.31				
					Mean	100.26				
TAP	1	4.0050	4.7971	8.8573	4.8523	101.15	0.9015	0.9003	0.1002	97.89 to 102.37
	2	4.0050	4.8366	8.8319	4.8269	99.80				
	3	4.0050	4.7530	8.7315	4.7265	99.44				
					Mean	100.13				

*Mean of three observations

TABLE – 34

OPTICAL CHARACTERISTICS OF TAPENTADOL HYDROCHLORIDE BY HPTLC METHOD

PARAMETERS	VALUES*
Detection wavelength (nm)	270
Beer's law limit (ng/µl)	100 - 600
Correlation coefficient (r)	0.9996
Regression equation (y = mx + c)	Y = 1.7701x + 19.6638
Slope (m)	1.7701
Intercept (c)	19.6638
LOD (ng/ µl)	4.3933
LOQ (ng/ µl)	13.3129
Standard error	0.6917

*Mean of six observations

TABLE – 35

QUANTIFICATION OF FORMULATION TAPOL-100 BY HPTLC METHOD

Drug	Sample No	Labeled amount (mg/tab)	Amount found (mg/tab)*	Percentage Obtained*	Mean (%)	SD	% RSD	SE	CI
TAP	1	100	99.9446	99.95	99.97	0.2196	0.2197	0.0061	99.74 to 100.20
	2	100	100.3275	100.33					
	3	100	100.0859	100.09					
	4	100	99.8097	99.81					
	5	100	99.7061	99.71					
	6	100	99.9101	99.91					

*Mean of six observations

TABLE – 36

INTRADAY ANALYSIS OF FORMULATION TAPOL-100 BY HPTLC METHOD

Drug	Sample No	Labeled amount (mg/tab)	Amount found (mg/tab)*	Percentage Obtained*	Mean (%)	SD	% RSD	SE	CI
TAP	1	100	99.9948	100.00	100.04	0.0551	0.0551	0.0061	99.90 To 100.18
	2	100	100.0105	100.01					
	3	100	100.1016	100.10					

*Mean of six observations

TABLE – 37

INTERDAY ANALYSIS OF FORMULATION TAPOL-100 BY HPTLC METHOD

Drug	Sample No	Labeled amount (mg/tab)	Amount found (mg/tab)*	Percentage Obtained*	Mean (%)	SD	% RSD	SE	CI
TAP	1	100	100.1016	100.10	100.03	0.0635	0.0635	0.0071	99.87 To 100.19
	2	100	99.9854	99.99					
	3	100	99.9917	99.99					

*Mean of six observations

TABLE –38

RECOVERY ANALYSIS OF FORMULATION TAPOL-100 BY HPTLC METHOD

Drug	Sample No	Amount present (ng/µl)	Amount added (ng/ µl)	Amount found* (ng/ µl)	Amount recovered (ng/ µl)	% Recovered	SD	% RSD	SE	CI
TAP	1	299.8920	240.0	541.5152	241.6232	100.68	0.5201	0.5193	0.0578	98.86 to 101.44
	2	299.8920	240.0	540.2158	240.3238	100.14				
	3	299.8920	240.0	539.0294	239.1374	99.64				
					Mean	100.15				
TAP	1	299.8920	300.0	602.0202	302.1282	100.71	0.3500	0.3488	0.0389	99.49 to 101.23
	2	299.8920	300.0	601.0033	301.1113	100.37				
	3	299.8920	300.0	599.9299	300.0379	100.01				
					Mean	100.36				
TAP	1	299.8920	360.0	658.8532	358.9612	99.71	0.2501	0.2502	0.0278	99.34 to 100.58
	2	299.8920	360.0	660.6610	360.7690	100.21				
	3	299.8920	360.0	659.7006	359.8086	99.95				
					Mean	99.96				

*Mean of three observations

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